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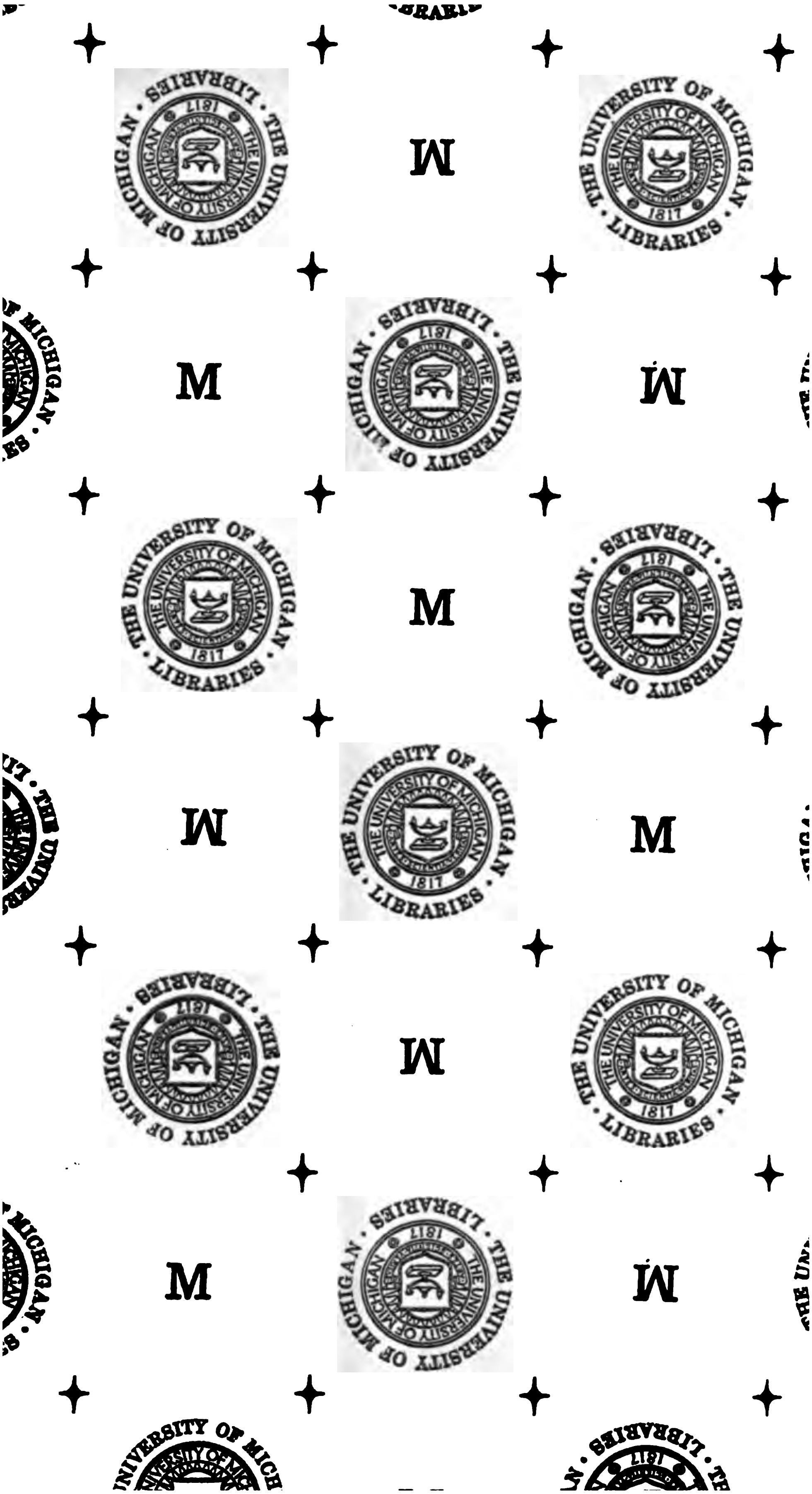

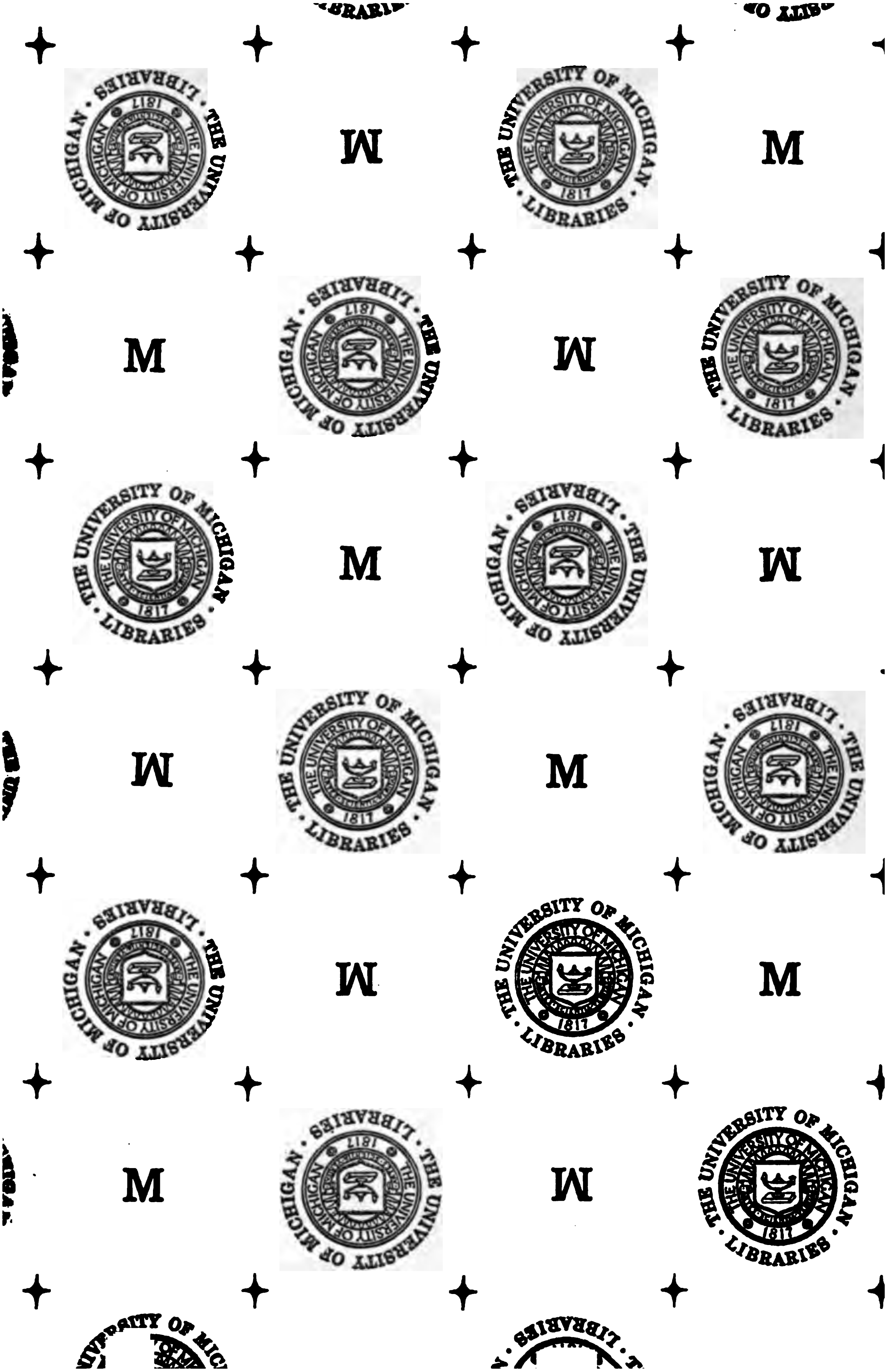
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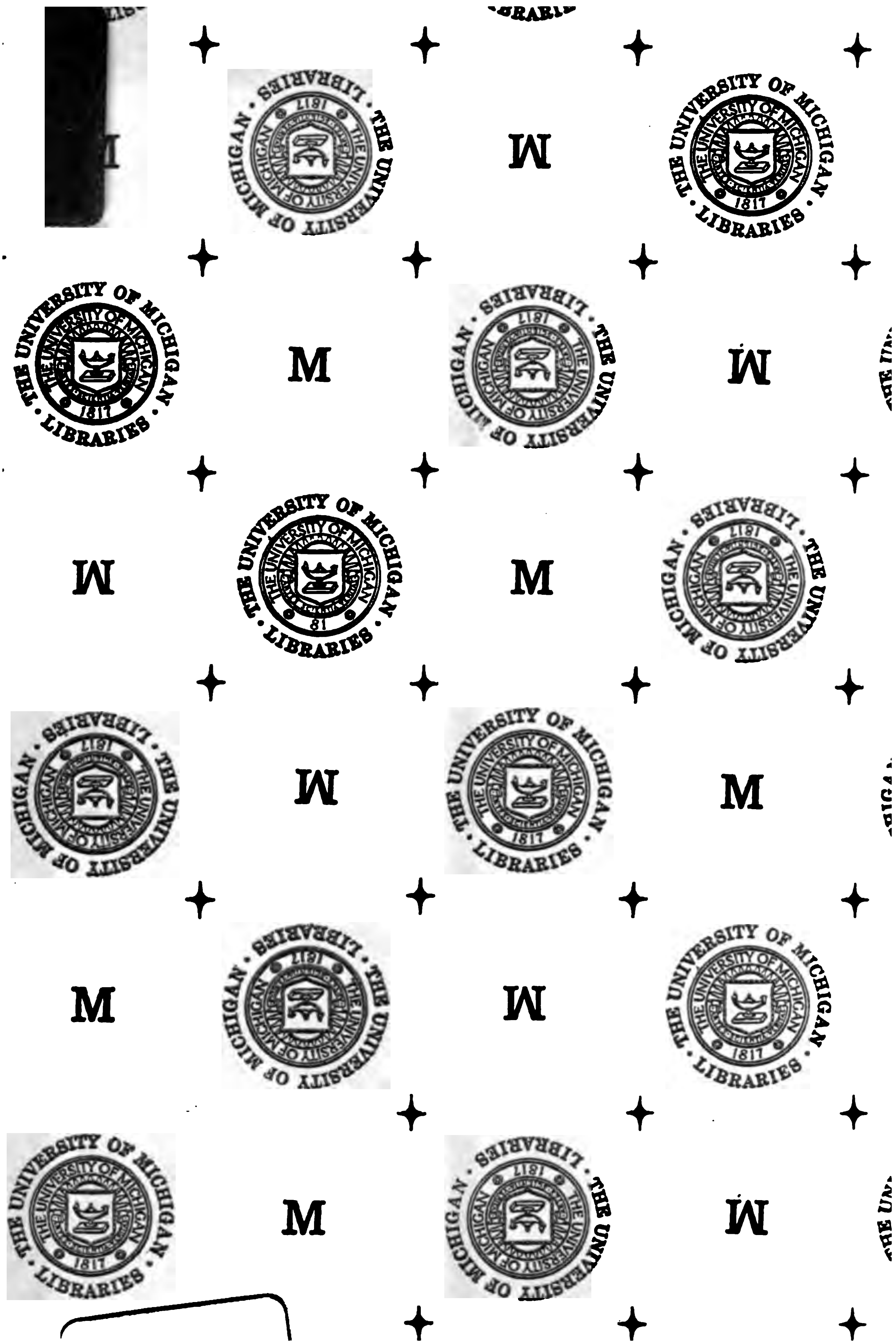

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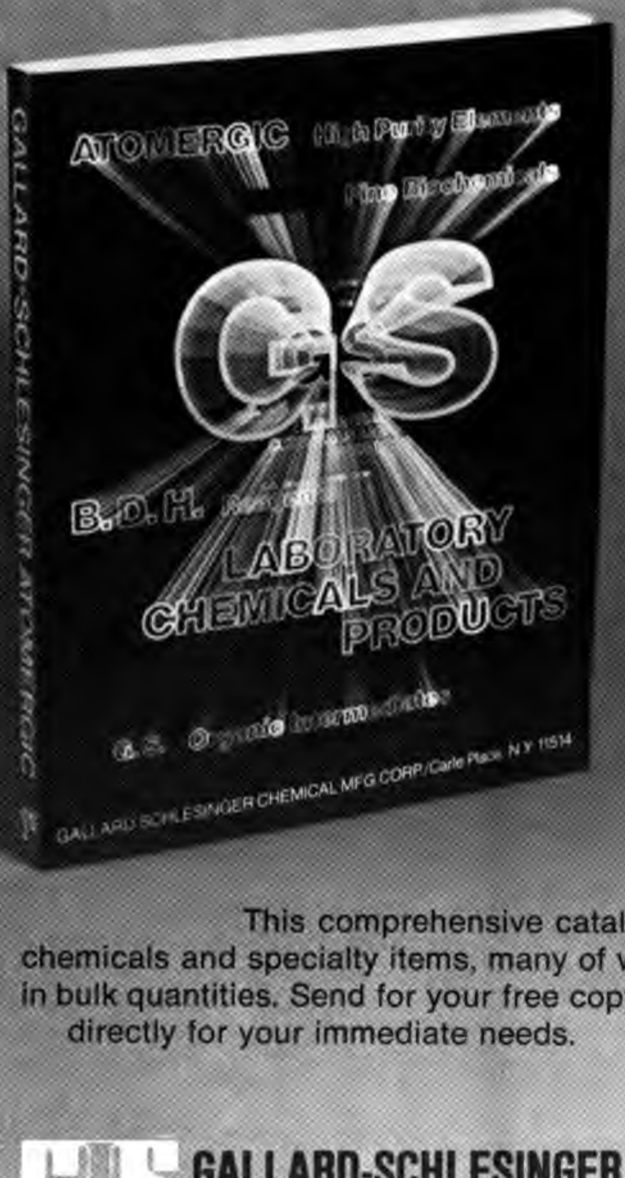
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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE

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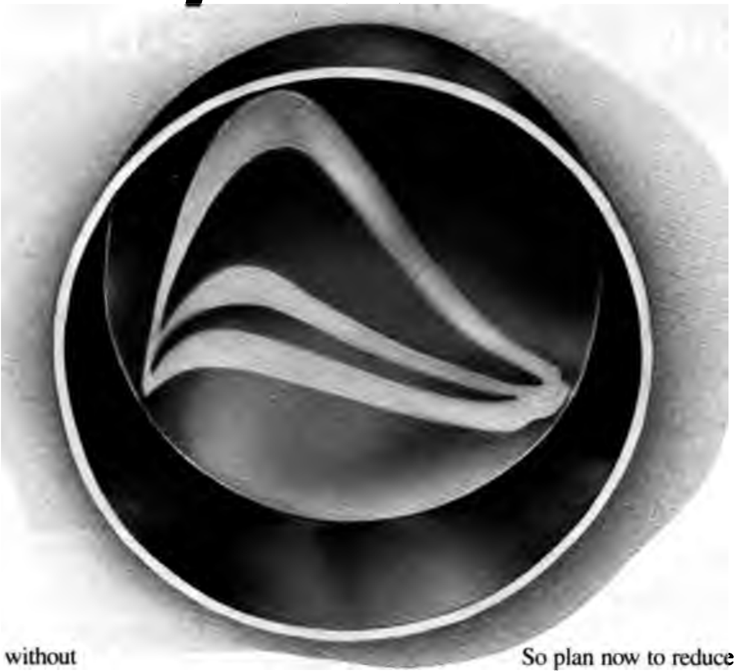

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GS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 1-5 (1976)

roxine and Triiodothyronine Influence Cultured Thyroid Cells: Alterations of Morphology and Mucosubstances (39314)

EDWARD SIEGEL[1]

nents of Radiology and of Medicine, University of Missouri Medical School, Columbia, Missouri 65201

important question bearing on the tion of the thyroidal hormones as well heir peripheral metabolism is whether d cells themselves respond to their ecretions. Utilizing the steer thyroid ie, it has been shown that exogenous ine does influence the formation of es and the synthesis of proteins (1). ultured cells used have been serially gated for many months during which t some aspects of differentiation were ved, including responses to thyrotro- SH) and the long-acting thyroid stim- (LATS), the synthesis of iodopro- and the maintenance of cytological ultrastructural features. Especially orthy among these specialized re- s are the highly ordered arrays resem- follicles that the dispersed cultured ssume following each subculture for st 3 months; this morphological pat- , we have shown, independent of TSH urther experimentation with this same e reveals that thyroxine (T_4) and triio- onine (T_3) affect morphological orga- n and the production of mucosub- s, the subjects of this communication.

rials and methods. Maintained as ayers, the cultured thyroid cells were in water-saturated incubators, gassed 5% air-5% CO_2 at 37°, using as the medium Eagle's MEM (3) supple- d with 10% fetal bovine serum and lin-streptomycin. They were subcul- ("passed") about every 2 weeks. The doubling time for this line is about 48 . The determinations being reported ere carried out on cells from the third vhich were allowed to overgrow onto f microscope slides contained in du- petri dishes and exposed for 96 hr to T_4 ($8.9 \times 10^{-7}\,M$), T_3 ($8.9 \times 10^{-7}\,M$), or as controls, to neither hormone. These preparations were fixed in Hanks' ethanol (1:1), followed by absolute alcohol. PAS-alcian blue staining at pH 2.5 (4) was carried out for demonstrating mucosubstances by exposing hydrated cells for 30 min to 1% alcian blue in 3% acetic acid, and rinsing in tap water for 5 min. The preparations were then oxidized for 10 min in 1% periodic acid and rinsed in tap water for 15 min. Finally, the slides were bathed in Schiff's Reagent for 60 min, 0.5% sodium bisulfite for 3 min, and washed in distilled water.

Results. Evaluation of the preparations indicates that even as late as the third pass, when the thyroid cell line was about 6-weeks old (more than 20 doubling periods having elapsed), the architecture and synthetic activity of these cells are strongly influenced by the exogenous T_4 and T_4. While both control and treated populations assume ordered arrays, many more of the cells exposed to the thyroidal hormones comprise semicircular or ringlike structures, reminiscent of partial or complete "follicles" (Fig. 1a, c). As compared to the influence exerted by T_4 (Fig. 1b) on these cultured thyroid cells, T_3 appears to be the more active in producing more numerous completed follicles possessing larger diameters and thicker septa (Fig. 1d).

As with the formation of follicles by these cultured cells, the synthesis of mucosubstances is also enhanced as a consequence of adding either thyroid hormone (Fig. 1a-d). The borders of the follicles of the populations exposed to T_4 or T_3 are frequently PAS-alcian blue positive and occasionally the "lumens" of T_3-treated cells contain large PAS plaques. As compared to the corresponding controls, the cells treated with either hormone have more alcian blue-staining granules associated with nuclei and pseudopodia. As judged by intensity of stain

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s investigation was supported by a research om the John A. Hartford Foundation, Inc., rk, New York.

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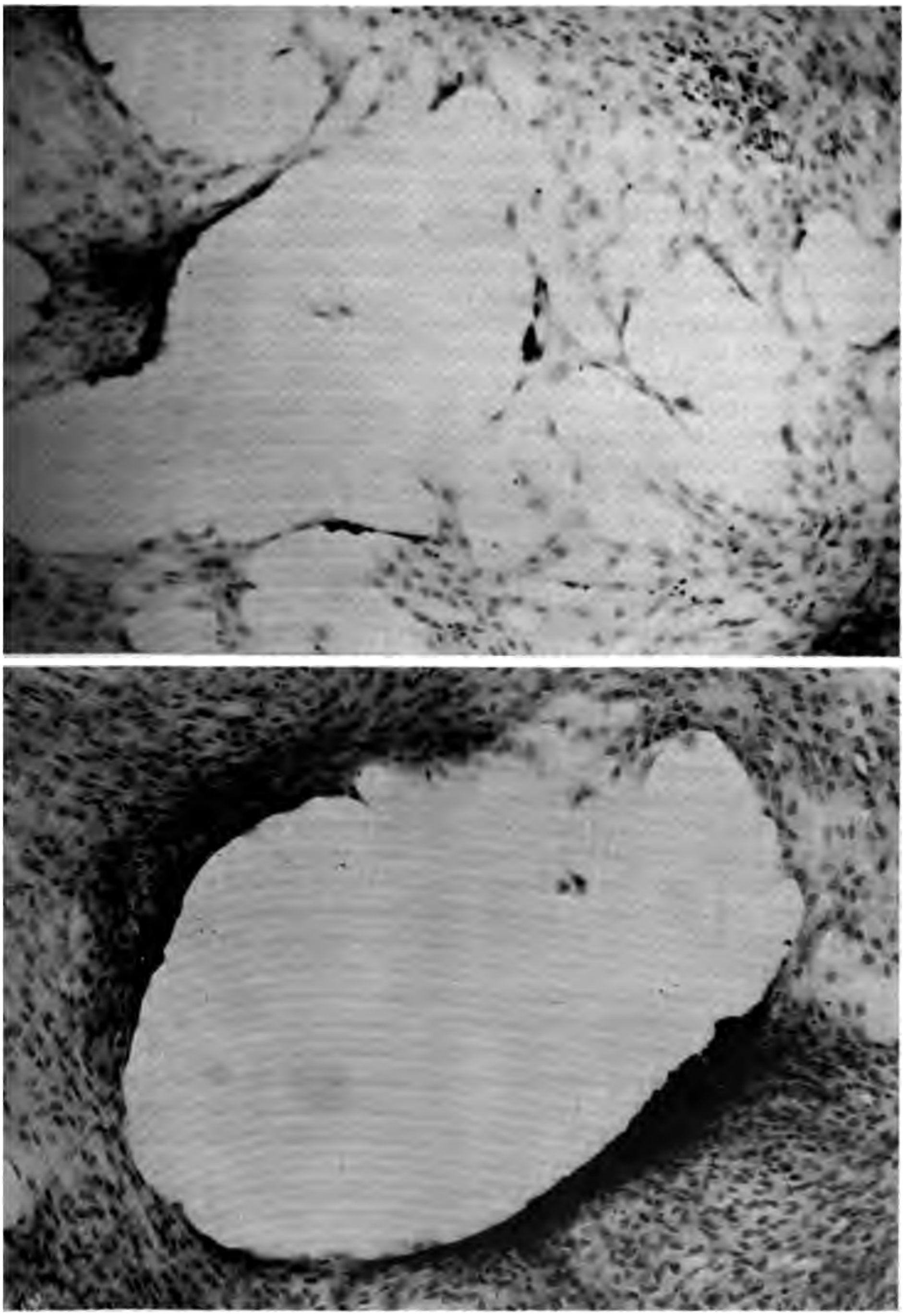
ndividual cells of the steer thyro

ances (× 50 8). (a) Control

ures are very rarely encountered

Follicular arrays are numerous

particles are more numerous



ver many of the follicular walls and adjoining regions. (c) T_3-treated cells: More complete follicles of eter with thickened "apical" walls appear throughout the field. Alcian blue and PAS staining over the eriphery and surroundings are frequently pronounced; occasionally (not illustrated) large PAS plaques r some "lumens." (d) T_3-treated cells: Another field (same magnification) illustrating a very large dimensions as well as the intensity of staining are much greater than those of T_4-treated cells.

and by the number and size of the dye-reaction particles, T_3 is once again more potent than T_4 (Fig. 1d).

Discussion. Like the formation of colonies and proteins shown previously (1), the cellular organization and the elaboration of mucosubstances are influenced by the thyroidal hormones, implying that T_4 and T_3 can act directly on the thyroid cell. For making the current pilot determinations, a hormonal titer (8.9×10^{-7} *M*) was chosen that had been shown earlier to evoke peripheral-like responses in the T-1 human kidney cell line (5). Obviously what yet needs characterizing in the steer thyroid cell line is the effects elicited by a broad range of T_4 and T_3 concentrations, especially those due to the lower hormonal levels that prevail *in vivo*. Even so, the findings made to date with the cultured thyroid cells point to lines of further investigation for ascertaining whether the thyroidal hormones have a role in intraglandular autoregulation, a subject of interesting conjecture (6–9).

As has been argued elsewhere (2), the serial propagation of cultured cells derived from isolated thyroid cells over many months, during which differentiated morphological and functional features are preserved, suggests that the adult steer cell may retain the potential for glandular morphogenesis and regeneration. In promoting the transformation of dispersed thyroid cells into highly ordered arrays, the thyroidal hormones may be expressing roles that correspond *in vivo* to those responsible for the morphological and embryonic development of the thyroid gland. Support for this notion comes also from the earlier observation already referred to (1), that T_4 and T_3 in enhancing colony formation by these cultured thyroid cells foster adhesion and aggregation which are requisite *in vivo* for follicles to exist.

A morphological transformation occurring over many passes and promoted by T_4 and T_3, the highly organized structures assumed by the isolated thyroid cells resemble follicles. The experimental evidence for this conclusion includes the localization of radioiodide and the release of labeled iodoproteins by these arrays in response to TSH and LATS; at the ultrastructural level it is noteworthy that TSH also stimulates rapidly the formation of electron-dense droplets and peripheral microvilli in these cultured thyroid cells (2), phenomena that closely simulate *in vivo* behavior (10). These morphological roles played by the thyroidal hormones, moreover, must be specific since they are not shared with TSH, which as has been noted, does not influence the development of folliclelike structures in the steer thyroid cell line nor in that derived from the adult porcine gland recently studied by others (11).

Assuming it also occurs *in vivo*, the influence of the thyroidal hormones on the synthesis of mucosubstances demonstrated here may bear at least on two aspects of thyroid gland metabolism. For the ontogenesis to occur that leads to the development of the thyroid gland, thyroid cells must mutually adhere and aggregate as has been remarked, a process known to require mucosubstances (12). Secondly, the effect of T_4 and T_3 on the elaboration of mucosubstances and proteins (1) may signify a relationship to thyroid hormone formation and secretion, possibly connected to autoregulation.

Summary. Further experimentation with the steer thyroid cell line indicates that the formation of "follicles" is enhanced by exposure to 8.9×10^{-7} *M* thyroxine (T_4) or triiodothyronine (T_3) for 96 hr. The production of mucosubstances by the cultured thyroid cells is also increased by treating with T_4 or T_3, effects demonstrable after staining with PAS-alcian blue at pH 2.5. It is suggested that these *in vitro* effects bear on morphological organization and synthetic activity of the thyroid gland as well as on autoregulation and intraglandular homeostasis that may occur *in situ*.

I thank Miss Jeanne Lammert, M.S., and Mrs. Elsie P. Siegel, M.S., for excellent technical assistance.

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Effect of Intrarenal Arterial Infusion of Magnesium on Renin Release in Dogs (39315)

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There is ample evidence that renin secretion is influenced by sodium load or concentration in the vicinity of the macula densa (8, 16). Other ionic species such as calcium (6, 10, 11) and potassium (1, 12) have been shown to affect renin secretion. The present research was undertaken to directly assess the effect of intrarenal arterial infusion of magnesium upon renin release in anesthetized dogs.

Methods. These experiments were performed on 15 adult (18–56 kg) mongrel dogs, fasted overnight, and anesthetized with sodium pentobarbital (30 mg/kg body weight, iv, with supplements given as required). Arterial, venous, left renal venous, and bilateral ureteral catheters were inserted; in four dogs, the right renal vein was also catheterized (4). 26-gauge needle, connected by a polyethylene catheter to a Harvard syringe pump, was introduced into the left renal artery near its aortic origin. Systemic arterial blood pressure was continuously monitored using a Narco pressure transducer and a multichannel Narco Physiograph.

Following this preparation, a priming solution of inulin and *p*-aminohippuric acid (5.0 g of inulin + 1.0 g of PAH in 100 ml of 150 m*M* NaCl, 1 ml/kg body weight) followed by a sustaining infusion (0.5 g of inulin + 0.5 g of PAH in 100 ml of 150 m*M* NaCl, 0.2 ml/min/kg body weight) were administered via the venous catheter. An infusion of 150 m*M* NaCl was begun in the renal artery at the rate of 0.6 ml/min, and after a 30–45-min equilibration period, two consecutive 10-min clearance periods were observed. Then $MgCl_2$ (0.8 *M*) was substituted for the 150 m*M* NaCl and infused at the same rate into the renal artery. Following a 30-min equilibration period, two additional 10-min clearance periods were observed. Then 150 m*M* NaCl was substituted for the $MgCl_2$ solution, and following a 30-min equilibration period, two recovery clearance periods were observed.

Arterial and renal venous blood was collected at clearance midpoints in heparinized, ice-cold plastic syringes and centrifuged at 4°. Ethylenediamine tetraacetate (1 mg) was added to duplicate 0.5-ml plasma samples which were then frozen until the renin assay could be done. A New England Nuclear Radioimmunoassay Kit (Boston, Mass.) was utilized for renin determinations. Plasma samples were thawed on ice, inhibitors of converting enzyme activity were added (10 μl of 1.7% dimercaprol in peanut oil and 10 μl of 6.6% 8-hydroxyquinoline in water, per ml of plasma), and the samples were incubated at 37°. Samples of 20 μl were removed at 1 and 2 hr incubation and angiotensin I concentration was determined by radioimmunoassay. Plasma renin activity was expressed as nanograms of angiotensin I per milliliter of plasma per hour incubation (ng A-I/ml/hr).

Sodium and potassium concentrations in the plasma and urine were determined by flame photometry using an internal lithium standard (Instrumentation Laboratories). Sodium and potassium excretion rates were calculated as the products of the urine concentrations (U_{Na} and U_K) and urine flow rate *(V)*. Magnesium was determined by atomic absorption spectrophotometry (Perkin-Elmer). Renal arterial Mg^{2+} concentration was calculated as the sum of the renal venous Mg^{2+} and the Mg^{2+} excreted/RPF. Inulin in urine and in trichloroacetic acid plasma filtrates was determined by the method of Harrison (5). Inulin clearance was calculated and used as the measure of glomerular filtration rate (GFR). PAH was determined by the method of Smith *et al.* (13). Renal plasma flow (RPF) was determined from the clearance of PAH corrected by its extraction. Paired Student's *t* test was used for the assessment of significance.

Results. The influence of intrarenal arterial $MgCl_2$ infusion on the plasma Mg^{2+} was determined in six dogs. The results are presented in Table I. The femoral arterial,

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LE I. EFFECT OF INTRARENAL ARTERIAL Mg^{2+} INFUSION ON PLASMA MAGNESIUM CONCENTRATION[a]

Mg^{2+} ration liter)	Control		Magnesium Infusion		Recovery	
	Clearance 1	Clearance 2	Clearance 3	Clearance 4	Clearance 5	Clearance 6
	1.29 ±0.04 (7)	1.23 ±0.06 (6)	2.98 ±0.28 (6)	4.10 ±0.58 (6)	3.19 ±0.43 (7)	3.03 ±0.42 (7)
ıal ve-	1.23 ±0.04 (7)	1.17 0.06 (6)	4.22 ±0.76 (6)	5.72 ±0.99 (6)	3.04 ±0.40 (7)	2.74 ±0.31 (7)
nal ve-	1.27 ±0.07 (3)	1.25 ±0.05 (2)	2.67 ±0.07 (3)	3.68 ±0.69 (4)	2.90 ±0.70 (3)	2.57 ±0.06 (2)
arterial ıted)	1.30 ±0.04 (6)	1.20 ±0.04 (6)	4.60 ±0.81 (6)	6.25 ±1.05 (6)	3.05 ±0.46 (6)	2.82 ±0.35 (6)

nesium was infused into left artery (0.4 mEq Mg^{2+}/min) during clearances 3 and 4. Renal arterial plasma centration was calculated as the sum of renal venous Mg^{2+} concentration and urinary Mg^{2+} concentration/ sults are presented as means ± SEM. Number of observations in parentheses. All values during s 3–6 are signficantly above control levels ($p < 0.005$).

enous, and calculated renal arterial ere significantly elevated throughout usion and recovery periods ($p <$. At these levels, we observed no ion of toxicity, such as a sharp drop in ressure or respiratory abnormalities. hown in Table II, there was a slight in mean arterial blood pressure and lasma flow throughout the experi- eaching significance ($p < 0.05$) dur- fourth clearance period. Magnesium d a significant diuresis ($p < 0.005$), o a significant natriuresis in both the < 0.005) and the fourth ($p < 0.01$) ce periods, without significantly ıg the plasma concentration of Na^+ or the GFR. The same changes were d in the right kidney, contralateral $MgCl_2$ infusion, but they were less ic and delayed.

effect of Mg^{2+} on renin secretion is rized in Table III. We observed no ant changes in arterial plasma renin during the experiment. The renal -arterial plasma renin activity (RV- as significantly increased during infusion ($p < 0.025$). Also, there increase in renin secretion rate, RPF), during $MgCl_2$ infusion (third clear- < 0.01; fourth clearance, $p <$ Renin secretion returned to base- ues during recovery.

ıssion. The present study extends the others who used rats (2), that Mg^{2+} tration produces a diuresis and na- in dogs. In those animals in which ı was monitored in both kidneys, the contralateral to the $MgCl_2$ infusion ıibited increased urine flow and sodium excretion. However, these responses were reduced and delayed from that seen in the other kidney (Table II).

Plasma Mg^{2+} concentrations on the order of 10 mEq/liter have been shown to produce respiratory abnormalities and sharp decreases in blood pressure (18). Although plasma Mg^{2+} never attained that level in the present experiments, a steady fall in blood pressure, which became significant after about 30 min of infusion, occurred throughout the experiment. Renal blood flow seemed to decrease in parallel with the changes in blood pressure, the fall becoming significant during the recovery period. Renin secretion increased significantly during Mg^{2+} infusion and rapidly returned to control levels in the recovery period. Although decreases in mean arterial blood pressure are known to stimulate renin secretion (16), it is doubtful that this mechanism was solely responsible for the observed renin stimulation. The increase in renin secretion was observed during the first Mg^{2+} clearance period, at which time blood pressure had not fallen significantly. Moreover, blood pressure was significantly reduced during recovery, when renin secretion had returned to normal.

The stimulation of renin during Mg^{2+} administration does not accord well with the macula densa theories proposed by Vander (16) or Thurau (14). According to these theories, Na^+ concentration, load, and transport play controlling roles in renin secretion. We found that $U_{Na}V$ increased (Na^+ reabsorption decreased) and renin secretion was stimulated during the infusion. However, during the recovery period, when

TABLE II. Effect of Intrarenal Arterial Mg^{2+} Infusion[a]

	Control		Magnesium infusion		Recovery	
	Clearance 1	Clearance 2	Clearance 3	Clearance 4	Clearance 5	Clearance 6
Mean arterial blood pressure (mm Hg)	138 ± 5 (15)	138 ± 5 (14)	133 ± 5 (15)	129 ± 5 (15)^{3}	127 ± 4 (15)^{3}	127 ± 4 (15)^{3}
Plasma sodium concentration (mEq/L)	145 ± 1 (15)	146 ± 1 (14)	145 ± 4 (15)	145 ± 4 (15)	145 ± 1 (15)	145 ± 1 (15)
Plasma potassium concentration (mEq/L)	3.8 ± 0.1 (15)	3.8 ± 0.1 (14)	3.8 ± 0.1 (15)	3.9 ± 0.1 (15)	3.9 ± 0.1 (15)	3.8 ± 0.1 (15)
Renal plasma flow (ml/min)						
Rt.	174 ± 40 (4)	145 ± 23 (4)	140 ± 12 (4)	128 ± 6 (4)	135 ± 11 (4)	127 ± 4 (4)
Lt.	167 ± 20 (14)	159 ± 20 (15)	157 ± 17 (14)	140 ± 16 (14)	131 ± 11 (15)^{2}	121 ± 9 (15)^{3}
Glomerlar filtration rate (ml/min)						
Rt.	40.7 ± 8.3 (4)	31.4 ± 7.8 (4)	33.1 ± 4.2 (4)	33.1 ± 4.2 (4)	40.7 ± 7.4 (4)	40.6 ± 6.0 (4)
Lt.	34.2 ± 3.4 (15)	32.6 ± 3.3 (14)	31.2 ± 3.1 (15)	28.6 ± 3.0 (14)	31.3 ± 3.2 (15)	29.9 ± 2.8 (15)
Urine flow rate (ml/min)						
Rt.	0.23 ± 0.08 (4)	0.51 ± 0.20 (4)	0.79 ± 0.21 (4)	0.86 ± 0.31 (4)^{2}	0.92 ± 0.17 (4)	1.10 ± 0.31 (4)^{1}
Lt.	0.46 ± 0.09 (14)	0.56 ± 0.11 (15)	1.34 ± 0.25 (15)^{4}	1.60 ± 0.23 (14)^{4}	1.34 ± 0.39 (15)^{4}	1.21 ± 0.33 (15)^{2}
Sodium excretion rate (mEq/min)						
Rt.	43 ± 5 (4)	43 ± 10 (4)	101 ± 38 (4)	101 ± 44 (4)	59 ± 20 (4)	71 ± 29 (4)
Lt.	63 ± 14 (15)	74 ± 19 (15)	148 ± 41 (15)^{4}	156 ± 38 (15)^{3}	114 ± 42 (15)^{1}	108 ± 38 (15)

[a] Magnesium was infused into the left renal artery (0.4 mEq Mg^{2+}/min) during clearances 3 and 4. Results are expressed as means ± SEM. Number of observations in parentheses. Signficance: ^{1} $p < 0.05$; ^{2} $p < 0.025$; ^{3} $p < 0.01$; ^{4} $p < 0.005$.

renin secretion had returned to control levels, plasma Mg^{2+} and $U_{Na}V$ were still elevated. Thus, the renin response was transient but any presumed stimulus should still have been operative. A simiar transience in response to Ca^{2+} might explain why some groups report a stimulation of renin secretion by Ca^{2+} (6, 10, 11, 19), while others find little or no effect (7, 9, 17).

It is difficult to reconcile these results with the observations of Cantin *et al.* (3). These workers report an increase in electrondense juxtaglomerular granules in Mg^{2+} deficiency. Since a good correlation has been demonstrated between pressor activity of renal extracts and the number of granules in JG cells (15), this suggests that Mg^{2+} might have been expected to decrease, rather than increase, renin secretion.

TABLE III. EFFECT OF INTRARENAL ARTERIAL Mg^{2+} INFUSION ON RENIN[a]

	Control		Magnesium infusion		Recovery	
	Clearance	Clearance 2	Clearance 3	Clearance 4	Clearance 5	Clearance 6
Arterial plasma renin (ng A-I/ml/hr)	16.8 ± 3.9 (15)	17.1 ± 4.2 (14)	17.5 ± 4.7 (15)	15.3 ± 4.2 (15)	13.1 ± 3.6 (14)	13.4 ± 3.8 (14)
Renal venous–arterial plasma renin (RV-A) (ng A-I/ml/hr)	4.01 ± 2.07 (15)	3.60 ± 2.26 (15)	6.86 ± 2.33 (15)[2]	5.89 ± 2.42 (15)[2]	4.38 ± 1.58 (14)	5.56 ± 2.13 (14)
Renal secretion rate RPF (RV-A) (ng A-I/hr/min)	515 ± 340 (15)	446 ± 280 (15)	992 ± 341 (15)[3]	924 ± 412 (14)[2]	469 ± 158 (14)	491 ± 165 (14)

[a] Magnesium was infused into the left renal artery (0.4 mEq/min) during clearances 3 and 4. Results are expressed as means ± SEM. Number of observations in parentheses. All renin values are for the left kidney. Significance: [1] $p < 0.05$; [2] $p < 0.025$; [3] $p < 0.01$; [4] $p < 0.005$.

Summary. Magnesium chloride was infused into the renal artery of anesthetized dogs in order to determine its effect on renal function. Natriuresis and diuresis were observed during $MgCl_2$ infusion, but there appeared to be no effect on glomerular filtration rate (GFR), or plasma sodium or potassium concentrations. Although mean arterial blood pressure and renal plasma flow (RPF) decreased throughout the experiment, the fall was not significant until after stopping $MgCl_2$ infusion. A significant stimulation of renin secretion occurred during magnesium administration.

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ar-Induced Potential Difference and Short Circuit Current in Bullfrog Small Intestine: Effect of UO_2^{2+} (39316)

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ions inhibit active sugar transport in l cell species, including yeast (1, 2), ithelial cells of renal tubules (3), and iall intestine (4–7). These inhibitory are believed to arise from interaction en UO_2^{2+} ions and membrane ligands are implicated in sugar transfer (4–8). the active step in sugar translocation small intestine is generally believed occur across the luminal border of the al cells, the effect of UO_2^{2+} on mu- ransfer of D-glucose, D-galactose, 3- hyl glucose, and luminal fluid by iso- at small intestine was investigated by , Sanford, and Smyth (6, 7). These s found that mucosal UO_2^{2+}, depend- its concentration, had two effects. At 0^{-4} *M*, UO_2^{2+} inhibited mucosal glu- ransfer and glucose-dependent fluid r. Mucosal transfer of galactose and ethyl glucose were unaffected. UO_2 3 ^{3} *M* inhibited mucosal transfer, under c conditions, of all three sugars. results were interpreted (7) as indi- that there are two pathways for the al entry of glucose into the epithelial f rat intestine, a relatively UO_2^{2+}- ve pathway which is not shared by se, and a less sensitive pathway involves a common carrier for glucose her actively transported sugars.

pling between active sugar transport t m → s Na^+ transfer in the small ie is well known and has been widely ented (9–12). This coupling is re- in the occurrence of sugar-induced ses in transmural potential difference and short circuit current (I_{sc}) (13, hese electrical changes appear to in- transfer mechanism which is shared cose and other actively transported s (14). Hence, one may predict that, suitable conditions, UO_2^{2+} should in- ugar-induced increases in P.D. and I_{sc}. The present paper reports the results of a study designed to test this prediction.

Materials and Methods. These experiments were performed on isolated segments of bullfrog (*Rana catesbeiana)* small intestine mounted as flat sheets between the two halves of an Ussing chamber. Both halves of the chamber were filled with an oxygenated sodium sulfate medium. Details of the preparation and mounting of the intestinal tissue, the Ussing chamber, the measurement of P.D. and I_{sc}, and the method used to circulate and oxygenate the fluid in each half of the chamber have been given elsewhere (15, 16). The bathing medium used was a modification of the sodium sulfate Ringer solution previously employed in this laboratory (15). A major modification was the omission of phosphate. Initially, the basic medium had the following composition (in mEq/liter): Na^+ 102.4, K^+ 4, Ca^{2+} 1.8, SO_4^{2-} 53.2, gluconate$^-$ 1.8. In addition, 67.5 mmole/liter mannitol was included to make the medium approximately isosmotic with frog plasma. Since UO_2^{2+} displays a complex set of interactions with OH^- at neutral pH (8), all experiments were run at pH 5.5. The pH of the medium was adjusted to this value with dilute H_2SO_4. Earlier investigations in this laboratory (Gerencser and Armstrong, unpublished observations) had indicated that the response of P.D. and I_{sc} in isolated bullfrog small intestine to actively transported sugars was essentially unchanged between pH 5.5 and 7.0. A preliminary set of experiments in the present study confirmed this finding. UO_2^{2+} was incorporated in the medium as $UO_2(NO_3)_2$. When this was done, an osmotically equivalent amount of mannitol was omitted. Early in this study we noticed that when $UO_2(NO_3)_2$ was present in the fluid bathing one side only of the tissue, artifactual P.D.s was induced. That these were

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eserved.

probably streaming potentials (17) arising from different transmural mobilities of NO_3^- and SO_4^{2-} is suggested by the fact that their magnitude appeared to depend on the transmural $UO_2(NO_3)_2$ concentration gradient and their orientation depended on the side of the chamber to which $UO_2(NO_3)_2$ was added. Also, these potentials could be suppressed by incorporating an equivalent amount of NO_3^- (added as $NaNO_3$) in the medium which did not contain UO_2^{2+}. The following procedure was therefore adopted in later experiments. The tissue was first equilibrated in a medium which contained an amount of $NaNO_3$ equal to twice the molar concentration of $UO_2(NO_3)_2$ used later in the experiment (the latter was usually 1.3 or 2.6 m*M*). An amount of Na_2SO_4 equivalent to the $NaNO_3$ added was omitted from this medium and its mannitol concentration was adjusted to keep the total osmolality constant. When P.D. and I_{sc} had reached a steady state, the fluid in one or both halves of the chamber was removed and replaced by an equal vol (4.7 ml) of a medium containing $UO_2(NO_3)_2$. After 2-3 min this initial aliquot of UO_2^{2+} medium was removed and replaced. This procedure was then repeated. Following this, electrical recordings were resumed. During electrical recording, the tissue was maintained in the "open circuit" condition except for occasional interruptions to measure I_{sc}.

All chemicals used in this study were "Analytical Reagent" grade and all solutions were made in distilled water which had been further purified by two passes through a mixed bed ion exchanger. It has been found (18) that, under conditions approximating those employed in the present experiments, the enhancing effect of actively transported sugars and amino acids on P.D. and I_{sc} are solely due to the solute in the mucosal medium. Therefore, to avoid the imposition of osmotic gradients across the tissue, 50-μl samples of concentrated solutions of D-glucose, 3-*O*-methyl glucose, and L-valine were added directly to both sides of the Ussing chamber to give final concentrations of 11, 26, and 15 m*M*, respectively. These correspond approx to a relative concentration (19) of 4 in each case (18). All experiments were performed at 25 ± 0.5°.

Results and Discussion. Effect of UO_2^{2+} on sugar-induced P.D. and I_{sc}. Preliminary experiments showed that UO_2^{2+} concentrations below 1 mEq/liter had no significant effect on glucose-induced P.D. and I_{sc}. When the medium bathing both sides of the tissue contained 1.3 mEq/liter of UO_2^{2+}, inhibition of these parameters was consistently found. Further experiments showed that the inhibitory effect of UO_2^{2+} was observed only when this agent was present in the mucosal medium. Serosal UO_2^{2+} alone had no effect on glucose-induced P.D. or I_{sc}. Figures 1 and 2 show representative results from these experiments. Two experiments are illustrated in Fig. 1. In one of these glucose was added to the mucosal and serosal media following 50-min incubation of the tissue in a glucose-free medium, and P.D. and I_{sc} were allowed to reach maximal values. At this point the mucosal medium was removed and replaced by a medium containing 1.3 mEq/liter of UO_2^{2+} and 11 m*M* glucose. It is apparent that, following

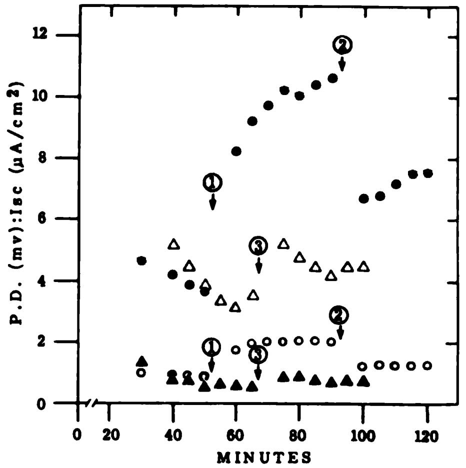
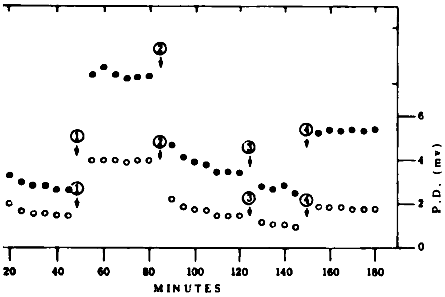


FIG. 1. Effect of mucosal UO_2^{2+} on glucose-induced P.D. and I_{sc}. Two experiments are shown. In one, glucose was added to both the mucosal and the serosal media at the time indicated by arrow (1). The responses of P.D. (O) and I_{sc} (●) to glucose were allowed to develop fully. Then, at the time indicated by arrow (2), the mucosal aspect of the tissue was exposed to 1.3 m*M* UO_2^{2+}. In the second experiment (P.D. - ▲; I_{sc} - △) the mucosal side of the tissue was exposed simultaneously at the time indicated by arrow (3) to glucose and UO_2^{2+}.



2. Effect of mucosal UO_2^{2+} on glucose-in-
'.D. (O) and I_{sc} (●). Arrow (1): glucose added
mucosal and serosal media; (2): both media
1 with glucose-free media; (3): 1.3 mEq/liter of
added to mucosal medium; (4): glucose added
media.

on of UO_2^{2+} to the mucosal medium,
was a substantial decrease in both
and I_{sc}. Although the degree of inhibi-
)f P.D. and I_{sc} was the same in any
experiment, the inhibitory effect of
+ in different experiments was some-
variable. In five experiments of the
ınder discussion the percentage inhibi-
ıf glucose-induced I_{sc} ranged from 21
(mean 42 ± 7 SEM). The average
tion of P.D. in these experiments was
6 percent.

: effect of mucosal UO_2^{2+} concentra-
ın the degree of inhibition of glucose-
ed P.D. and I_{sc} was not systematically
d. Three experiments in which the
+ concentration was 2.6 mEq/liter
results within the range quoted above
3 mEq/liter of UO_2^{2+}. Virtually com-
inhibition of glucose-induced increases
D. and I_{sc} was consistently observed
13 mEq/liter of UO_2^{2+}, but in most of
experiments, steady state P.D. and I_{sc}
ring addition of UO_2^{2+} were signifi-
less than their initial baseline values.
s shown previously (15) that, under
r conditions to those used in the pres-
udy, I_{sc} in the absence of transported
s reflects net m → s Na^+ transfer in
og small intestine. It may therefore be
ided that, in concentrations of the or-
13 mEq/liter, UO_2^{2+} inhibits the basic
ınism of net Na^+ transport in this tis-

inhibitory effect of UO_2^{2+} on glucose-induced P.D. and I_{sc} was quite rapid. This is illustrated in the second experiment shown in Fig. 1. In this experiment glucose and UO_2^{2+} (1.3 mEq/liter) were added simultaneously to the mucosal medium. It is apparent that, under these conditions, the increase in P.D. and I_{sc} was very slight and rapidly reached a steady state. This is consistent with the suggestion (4) that, in small intestine, as in other tissues (8, 20), the inhibitory effects of UO_2^{2+} involve an interaction between this ion and anionic ligands (specifically, phosphoryl and/or carboxyl groups) in the epithelial cell membrane. The fact that glucose induced increases in P.D. and I_{sc} are only observed when this sugar is added to the medium bathing the mucosal surface of the epithelial cells (9, 12, 19) suggests that these ligands are located in the brush border membrane.

In the experiment represented by Fig. 1, P.D. and I_{sc} following addition of glucose and of UO_2^{2+} always reached a higher steady state level than their initial values under control conditions. Hence one could argue that these experiments do not provide unequivocal evidence that UO_2^{2+} specifically inhibits the glucose induced moiety of P.D. and I_{sc}. Similar results to those shown in Fig. 1 could be predicted on the assumption that UO_2^{2+} inhibits P.D. and I_{sc} in the absence of glucose or partially inhibits both the glucose-dependent and glucose-independent P.D. and I_{sc}. Fig. 2 shows one of a series of experiments designed to test this possibility. In this experiment glucose was added to both the mucosal and the serosal medium (arrow 1) and the expected increases in P.D. and I_{sc} were allowed to reach stable values. At this time (arrow 2) both media were replaced by a glucose-free Ringer solution, and P.D. and I_{sc} were permitted to decline to values close to those observed initially. Following this, the mucosal medium was replaced by a medium containing 1.3 mEq/liter of UO_2^{2+} (arrow 3 of Fig. 2). Finally (arrow 4), glucose was again added to both the mucosal and the serosal medium. It is apparent from Fig. 2 that whereas mucosal UO_2^{2+} had virtually no effect on P.D. and I_{sc} in the absence of glucose, the response of these parameters to glucose was markedly reduced when UO_2^{2+} was present in the mucosal medium. Control experiments showed that, following an initial stimulation of P.D. and I_{sc} by glucose and reexposure of the tissue to a glucose-free medium, a second addition of glucose elicted an undiminished response to these parameters. Fig. 2 clearly indicates that the inhibitory effect of UO_2^{2+} at a concentration of 1.3 mEq/liter is restricted to the glucose-evoked increases in P.D. and I_{sc}.

Figure 3 indicates that, as expected, the inhibitory effect of mucosal UO_2^{2+} on glucose-induced changes in P.D. and I_{sc} extends to other actively transported sugars and sugar analogs which share a common transport pathway with glucose (14). It is apparent from this figure that the increases in P.D. and I_{sc} elicited by 3-*O*-methyl glucose are rapidly and strongly inhibited when 1.3 m*M* UO_2^{2+} is added to the mucosal medium. As was found with glucose, serosal UO_2^{2+} at this concentration did not inhibit the increases in P.D. and I_{sc} elicited by 3-*O*-methyl glucose.

Frizzell and Schultz (21) have presented evidence that anionic groups in the mucosal cell membrane (most probably, in these authors' opinion, carboxyl groups, although they do not rule out phosphoryl groups as a possibility) are involved in the influx of L-alanine across the brush border of isolated rabbit ileum and have shown that, at pH 5, alanine influx in this tissue is markedly reduced by 1.0 m*M* mucosal UO_2^{2+}. Further, Foulkes (20) had shown that the transport of amino acids by rabbit kidney *in vivo* is inhibited by UO_2^{2+} poisoning, though, in this instance, the inhibitory effect appears to be located in the peritubular rather than the luminal cell membrane. Because of these findings, we examined the effect of UO_2^{2+} on valine-evoked increases in P.D. and I_{sc} (18). In these experiments mucosal UO_2^{2+} (1.3 mEq/liter) had no effect on the P.D. and I_{sc} induced by 20 m*M* valine (Fig. 4). Similarly, serosal UO_2^{2+} at the same concentration did not affect these parameters.

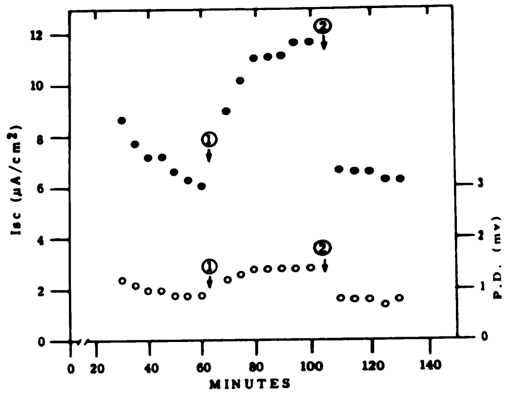


Fig. 3. Inhibition of P.D. (○) and I_{sc} (●) elicited by 35 m*M* 3-*O*-methyl glucose (added at arrow 1) by mucosal UO_2^{2+} (1.3 mEq/liter, added at arrow 2).

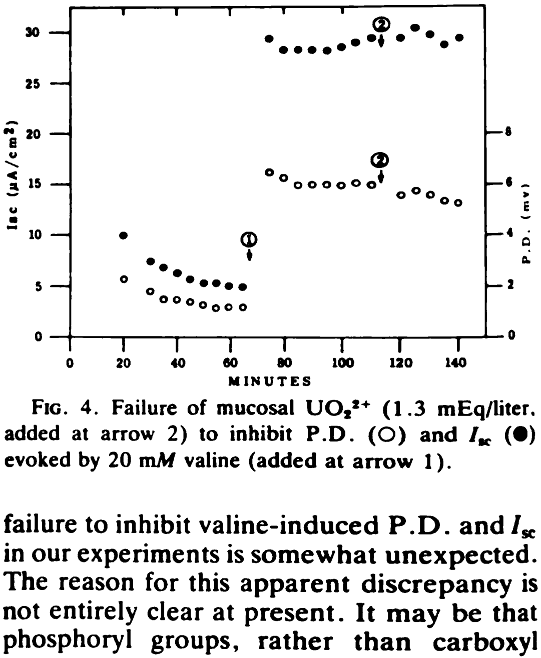


Fig. 4. Failure of mucosal UO_2^{2+} (1.3 mEq/liter, added at arrow 2) to inhibit P.D. (○) and I_{sc} (●) evoked by 20 m*M* valine (added at arrow 1).

In view of the well-known coupling between the transport of sodium and that of amino acids in the brush border of the small intestine (9) and since mucosal UO_2^{2+} inhibits alanine transport in this tissue (21), its failure to inhibit valine-induced P.D. and I_{sc} in our experiments is somewhat unexpected. The reason for this apparent discrepancy is not entirely clear at present. It may be that phosphoryl groups, rather than carboxyl groups, are implicated in intestinal sugar uptake. The former have been shown to possess a higher binding affinity for UO_2^{2+} than the latter both *in vitro* and *in vivo* (8). Hence sugar-evoked increases in P.D. and I_{sc} might be more sensitive to UO_2^{2+} inhibition than the changes induced in these parameters by amino acids. In the light of the results reported by Frizzell and Schultz (21) this view would seem to require a lower sensitivity of amino acid transport to UO_2^{2+} inhibition in bullfrog small intestine than in rabbit ileum. Alternatively, significant inhiition of amino acid influx without any obrvable decrease in amino acid-induced .D. and I_{sc} could occur if the coupling ratio r the cotransport of Na^+ and valine across e brush border were altered by mucosal O_2^{2+}. It is known that the stoichiometry of a^+/alanine cotransport is sensitive to a variety of factors including the pH of the bathg medium (21) and luminal Na^+ concenration (22). Whatever the origin of this iscrepancy, it seems clear that, under the onditions of our experiments, UO_2^{2+} selecively inhibits sugar-induced P.D. and I_{sc} in solated bullfrog small intestine and, by imlication (6, 7, 9), also selectively inhibits nucosal uptake of sugars by this tissue unler these conditions.

Summary. UO_2^{2+} 1.3 m*M* added as $O_2(NO_3)_2$ to the mucosal solution consistntly inhibited the P.D. and I_{sc} evoked by 11 m*M* glucose and 35 m*M* 3-*O*-methyl gluose across isolated strips of bullfrog small ntestine bathed by symmetrical Ringer soutions in which SO_4^{2-} was the major anion. The average degree of inhibition in the presnce of glucose was 42 ± 7 (SEM) percent. .D. and I_{sc} in the absence of transported olutes were not significantly altered by muosal UO_2^{2+} at this concentration. Increasng the mucosal UO_2^{2+} concentration to 2.6 n*M* did not significantly increase its inhibiory action on glucose-evoked P.D. and I_{sc}. urther increasing the UO_2^{2+} concentration o 13 m*M* completely inhibited glucose-inluced P.D. and I_{sc} but also markedly reluced these parameters in the absence of lucose. Serosal UO_2^{2+} (1.3 m*M*) had no ffect on the P.D. and I_{sc} evoked by glucose nd 3-*O*-methyl glucose. It is suggested that he inhibitory action of UO_2^{2+} involves inding of this ion to anionic sites located in he apical membrane of the absorptive cells. Mucosal or serosal UO_2^{2+} (1.3 m*M*) had no effect on the P.D. and I_{sc} elicited by 20 m*M* valine, indicating that under the conditions of these experiments UO_2^{2+} selectively inhibits sugar-induced P.D. and I_{sc} and, by implication, mucosal sugar uptake.

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Glucose Tolerance, Insulin and Catecholamine Levels in Germfree Rats[1] (39317)

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In the many studies which employ germfree (GF) or gnotobiotic rodents to control the microbial variable, a matter of prime concern is the comparability of their functional and metabolic parameters to those of conventional (CV) animals. GF rats have shown lower cardiac output and metabolic rate than CV rats (1, 2). Thyroid function appears retarded in its development with age, although thyroxine levels in mature GF rats are comparable to those found in their CV counterparts (3). In addition, the microvasculature of GF rats was found to be relatively insensitive to the action of catecholamines and certain bioactive peptides (4, 5). Since definite differences in metabolic rate (1, 2, 6) and metabolic enzyme activity (3, 7) between GF and CV rats have been established, further studies to determine the factors that control homeostasis in the GF rat appear indicated.

Although in their earlier research, Desplaces *et al.* had suspected the GF rat of a "prediabetic state," later studies showed no evidence of impaired glucose tolerance (8). Wiech *et al.* (9), on the other hand, found elevated fasting plasma glucose levels in the GF rat, delayed and decreased insulin secretion, and reduced glucose clearance; these findings were associated with elevated plasma triglyceride levels. To resolve these ambiguities we have determined plasma glucose levels and glucose tolerance in GF and CV Lobund rats of Wistar origin, relating them to plasma insulin levels before and after intravenous glucose load and to the possible implications of the relative catecholamine refractoriness established for the GF animal (4).

Materials and Methods. The fifty and 100-day-old GF and CV male rats of Wistar origin (LOB(Wi)h) used in this study had been reared on sterilized L-485 (grain-soy) diet (10). Germfree animals were maintained in flexible plastic isolators (11), and CV controls were kept in the open colony room.

The animals were fasted for 20 hr preceding glucose tolerance tests. All samples were obtained between 9 and 11 AM. The GF rats were removed from the isolator prior to the experiment. Under prolonged anesthesia (35 mg sodium pentobarbital[2] per kg body weight, given ip), a cannula of PE 50 polyethylene tubing was inserted into the carotid artery. Blood samples collected for glucose determination and for the insulin immunoassay were 0.2 and 0.5 ml, respectively.

Approximately 8 animals per age and status group were used to establish the glucose tolerance curves. In additional 100-day-old GF and CV animal groups of similar size, both glucose and insulin were determined initially. After the initial samples were taken, all animals received 125 mg of glucose per 100 gm of body weight injected through the saphenus vein. Blood samples were obtained at 5, 30, and 60 min following glucose administration for glucose tolerance. In the additional 100-day-old animals, second samples for both glucose and insulin assays were taken only at 30 min. After each collection a small amount of heparin (0.05 ml of 1:10 diluted sodium heparin) was injected into the tubing to prevent coagulation. Between collections the artery was clamped off. Blood glucose was determined by the glucose oxidase method (12).[3]

Immunoreactive insulin was measured by a double-antibody technique of Hales and Randle (13) using an Amersham/Searle[4] ra-

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[1] These studies were supported by NIH Grant No. RR0640.

[2] Nembutal, Abbott Laboratories, Chicago, Illinois.

[3] Glucostat, Worthington Biochemical Corp., Freehold, New Jersey.

[4] Amersham/Searle Corp., Arlington Heights, Illinois.

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ve insulin kit. Rat insulin obtained NOVO Industries[5] was used as a rd.

separate experiment plasma catechol- levels were assayed in untreated GF V rats by the Anton and Sayre (14) oxyindole method with slight modifi- (15). To avoid release of catechola- under the influence of pentobarbital, ts (100-day-old) were anesthetised by inhalation of Fluothane[6] GF rats nesthetized within the isolator. Blood llected immediately by cardiac punc- he plasma catecholamines were ana- fluorometrically utilizing a Farrand fluorometer[7] with 5-mm slits in the g monochromator and 20-mm slits in alyzing monochromator. Photomulti- mplification was maximal.

lts and discussion. There are contra- reports in the literature regarding ponse of GF rats in the glucose toler- st. Desplaces *et al.* (8) reported that d CV rats exhibited a similar toler- oral glucose loading, although the glucose level of the germfree animal nsistently lower. On the other hand, *et al.* (9) reported that the fasting glucose level was higher in the GF d remained elevated up to 3 hr after icose administration. Since a number rs (age, anesthetic, route of glucose stration) can affect the glucose toler- st (8, 16, 17), we have administered intravenously so that differences in absorption or stress from esopha- tubation would be minimized. Des- *et al.* (8) had demonstrated that pen- ital anesthesia as such has little influ- n blood glucose levels of the Wistar his was more recently confirmed by *et al.* for a 25-mg/kg body weight vel (18). Only at a 50-mg/kg level a slight but significant increase in glucose levels be observed in the e-Dawley rats used in their study. on, however, found no influence t this level on insulin release after nous glucose administration to Sprague–Dawley rats, although 10-min glucose levels were 8% higher than in anaesthetized controls (19). No important effect would be expected from the 35-mg/kg dose used in the present investigation.

The data from 50- and 100-day-old GF and CV rats are summarized in Table I. Since comparison of glucose levels of the 100-day-old GF and CV rats who had additional blood withdrawn for insulin determination with those rats in which only glucose was determined, had shown no influence of the additional sample taking, these data have been combined. The glucose tolerance profiles of GF and CV rats appear quite similar, and comparable to those described for CV rats by DeSantis *et al.* (20). Only the average 5-min value of the 50-day-old CV rats was found slightly but significantly below the averages of the three other 5-min groups. Wiech has reported fasting plasma glucose levels of GF 50-day-old Fischer rats to be higher than CV levels (9). This was not confirmed by the present study.

Plasma insulin levels before and 30 min after glucose administration to 3-month-old GF and CV Lobund Wistar rats are given in Table II. The data for the CV animals fall in the same range as those of Aranda *et al.* (21). Plasma insulin levels in the GF rat tend to be somewhat lower than in the CV animal. However, insulin mobilization in the GF rats appears adequate for the control of plasma glucose levels. The data demonstrate a percentage increase in plasma insulin concentration during the 30 min after load that is comparable to that in CV rats. In both GF and CV rats the percentage increase in insulin concentration matches the increase in blood glucose. Clearance rate and final blood glucose level (1 hr after glucose administration) in GF rats again equal those observed in their CV counterparts (Table I).

It is well documented that catecholamines affect insulin secretion (22). Recently Baez and Gordon (4) have reported that the microvasculature of the GF rat is refractory to catecholamines, and have related this observation to a material (α-pigment) produced mainly in the GF cecum (5). If this phenomenon were to be explained on the basis of a competition for catecholamine receptor sites by circulating α-pigment, it is conceiv-

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O Industries, Copenhagen, Denmark.

st Laboratories, Inc., New York, New York.

nd Optical Co., Inc., Mt. Vernon, New

TABLE I. Blood Glucose Levels (mg/100 ml) during Glucose Tolerance Test.[a]

Age (days)	Status	Body wt[b] (g)	Time after administration of glucose (min) 0	5	30	60
50			(7)	(7)	(7)	(7)
	Germfree	166 ± 8	123 ± 4	418 ± 15	224 ± 8	185 ± 6
	Conventional	149 ± 20	112 ± 5	359 ± 7[c]	236 ± 6	184 ± 7
100			(17-20)[d]	(8)	(17-20)[d]	(8)
	Germfree	290 ± 28	123 ± 5	433 ± 27	220 ± 8	177 ± 9
	Ratio 30 min/0 min				1.88 ± 0.09[e]	
	Conventional	307 ± 23	122 ± 4	400 ± 15	219 ± 6	183 ± 1
	Ratio 30 min/0 min				1.80 ± 0.06[e]	

[a] Mean ± SE for the number of animals indicated in parentheses.

[b] Mean ± SD for that status and age group.

[c] Significantly different from all other 5-min groups.

[d] Includes animals used for plasma insulin determination.

[e] Average ratio ± SE of individual values.

TABLE II. Plasma Insulin Concentrations (μU/ml) before and 30 Min after iv Glucose Administration to 100-Day-Old Male Germfree and Conventional Rats.

Time (min)	Germfree	Conventional	P
0	33 ± 4.4[a] (11)	55.0 ± 8.8 (11)	0.08
30	63.8 ± 11 (7)	132 ± 22 (6)	0.03
Ratio 30/0[b]	1.8 ± 0.2 (7)	2.3 ± 0.4 (6)	NS

[a] Mean ± SE for the number of animals indicated in parentheses.

[b] Average ratio ± SE of individual values.

able that plasma catecholamine levels would show a compensatory increase under GF conditions. However, the data in Table III, which compare well with values reported in the literature (23, 24), indicate no significant difference in plasma catecholamine levels between GF and CV rats. It is therefore concluded that in their function as modulators of carbohydrate metabolism, circulating catecholamines would affect GF and CV rats in a comparable way.

TABLE III. Plasma Catecholamine Levels in 100-Day-Old Germfree and Conventional Rats.[a]

Status	Epinephrine (μg/liter)	Norepinephrine (μg/liter)
Germfree	5.76 ± 0.66 (12)	7.13 ± 0.95 (11)
Conventional	6.93 ± 0.74 (10)	6.98 ± 0.79 (11)

[a] Mean ± SE for the number of animals indicated in the parentheses.

Insofar as plasma insulin levels in the GF rat tend to be somewhat below those found in the conventional animal without affecting glucose tolerance, the data appear to agree with views expressed by Desplaces *et al.* (8). Recently Tolbert and Fain have again emphasized the independence of the two gluconeogenic hormones, epinephrine, and glucagon (25). Epand and Douglas (26) have studied the interrelationship between glucagon and insulin, and have shown that when glucagon levels are lowered artificially, normal fasting glucose levels and glucose tolerance will be achieved at lower insulin levels. It could be speculated that the present finding of normal fasting levels and tolerance curves in the presence of somewhat lower insulin concentrations may be caused by a correspondingly lower glucagon output of the GF Lobund Wistar rat. This needs to be investigated. However, the present data indicate that insulin levels in the GF rat are adequate to assure normal plasma glucose and glucose tolerance. There is therefore no reason to believe that insulin insufficiency plays any role in the syndrome of metabolic anomalies demonstrated by the germfree rat.

Summary. Glucose was administered intravenously to 50- and 100-day-old GF and CV rats. Fasting blood glucose levels in GF and CV rats were found to be comparable. Glucose tolerance tests showed that GF and CV rats clear glucose from the blood at a

milar rate. Although insulin concentraons in 100-day-old GF rats tended to be mewhat lower than in CV rats, the perntage increase during the 30-min period ter glucose administration was similar, d matched the increase in blood glucose. evels of plasma catecholamines were anazed fluorometrically and were found to be mparable in 100-day-old GF and CV rats. was concluded that insulin insufficiency lays no role in the syndrome of metabolic omalies demonstrated by the germfree t.

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Purification of Conjugated Bilirubin: A New Approach Utilizing Albumin-Agarose Gel Affinity Chromatography (39318)

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Bilirubin, an organic anion and breakdown product of heme, is excreted chiefly by the liver by a process involving uptake by the hepatocyte and subsequent conjugation to form a more polar compound (1). The availability of purified preparations of bilirubin, especially of radiolabeled bilirubin, has greatly facilitated studies of its metabolism (1-5). Study of the transport of bilirubin from blood to bile, however, must also include consideration of the mechanisms for excretion of bilirubin conjugates. Such study has been limited because of difficulty in obtaining conjugated bilirubin free of significant quantities of contaminants (6-19).

Recent studies of albumin-agarose gel affinity chromatography have revealed that the gels have a high affinity for bilirubin and a relatively low affinity and capacity for tri- and dihydroxy bile acids (20, 21), suggesting that this method might effectively separate bilirubin conjugates from other components in bile. We describe here, a rapid and relatively simple method for purification of conjugated bilirubin from bile.

Materials and methods. Albumin-agarose gel was prepared by a modification of the cyanogen bromide method (20, 22), and packed in small columns of 0.9-cm i.d. Bile acids were quantitated in bile and column eluates by gas-liquid chromatography (23), cholesterol by the method of Zak *et al.* (24), and phospholipids by a modification of the method of Chen *et al.* (25, 26).

Absorption spectra were obtained with a Beckman Acta III spectrophotometer, and fluorimetric analyses with a Farrand Mark I spectrofluorimeter.

Tritium was quantitated by adding up to 0.5 ml of sample to 15 ml of Aquasol (New England Nuclear) and by counting in a liquid scintillation counter (Nuclear Chicago). All samples were recounted after addition of [^{3}H]toluene (New England Nuclear) and results were corrected for background and quenching.

Bilirubin conjugates were quantitated after diazotization with ethyl anthranilate according to the method of Van Roy and Heirwegh (27). The resultant azopigments were separated by thin-layer chromatography (28) and bilirubin conjugates were identified (28, 29). Multiple determinations of the proportions of monoglucuronide and diglucuronide in a single bile specimen agreed within 1% of the mean. Presence of conjugated bilirubin was confirmed by Weber-Schalm fractionation (30) and by thin-layer chromatography performed on pigment applied directly to a silica gel plate and developed with chloroform (31).

Glucuronic acid content of conjugated bilirubin preparations was determined by a modification of the carbazole reaction (32) after hydrolysis of the ester linkage by incubation with 0.2 *N* NaOH.

Bile collection. Biliary cannulae were inserted into male Wistar rats under light ether anesthesia. The rats were kept in a restraining cage within an incubator; body temperature was maintained at 37^c. Starting 2 hr after insertion of the cannulae, when a constant rate of bile flow had been established, bile was collected in the dark under ice and frozen until used. Bile containing radiolabeled conjugated bilirubin was collected in a similar fashion. A jugular cannula was inserted simultaneously with the construction of the bile fistula, and 100 μCi of 2,3-^{3}H delta-aminolevulinic acid (33,000 mCi/mmole) (33) was injected via the cannulated jugular vein 2 hr after insertion of the cannulae.

Preparation of purified bilirubin conju-

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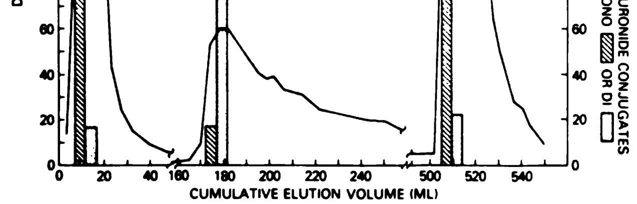
Rat bile, 8 to 10 ml, was applied to an lbumin-agarose column equilibrated).02 *M* phosphate buffered saline at pH 7.0. The column was washed 50 ml of PBS at pH 7.0 at a rate of 3 ı. Elution in 2- to 4-ml aliquots was ned by distilled water followed by v/v) ethanol in water. The water and l eluates were lyophilized, dissolved ıl of 0.02 *M* PBS at pH 5.8, and ed twice with 2 vol of chloroform to unconjugated bilirubin. Protein, t as a trace contaminant, was re- by applying the resulting PBS solu- Sephadex G-25 (2 × 20 cm) which en equilibrated with PBS at pH 7.0. of the yellow pigment, representing ı-free conjugated bilirubin, remained to the column, whereas protein and ı-bound pigment eluted with 150 ml at pH 7.0. Residual pigment on the ı was rapidly eluted with a small vol- distilled water. The aqueous solution ›philized, dissolved in 1 ml of 0.02 *M* pH 7.0, applied to the Sephadex G- ımn, washed with 150 ml of 0.02 *M* pH 7.0, eluted with approx 10 ml of d water, and lyophilized. Further pu- on of these preparations to remove hospholipid contamination was per- by extraction of the aqueous solu- conjugated bilirubin three times with 25 vol total of chloroform-methanol (2:1).

Results. Figure 1 illustrates the elution pattern of radiolabeled bile from an albumin-agarose affinity column. Approximately 20–30% of labeled material did not bind and washed through with PBS. Distilled water eluted a fraction of pigment; 50% ethanol eluted a second fraction. The elution pattern of unlabeled bile was identical to that of radiolabeled bile.

Thin-layer chromatography of pigment from both the water and ethanol eluates (31) revealed that only conjugated bilirubin was present in each. Following diazotization, thin-layer chromatography of the ethyl anthranilate azopigments of conjugated bilirubin in bile and in each of the eluates was performed (Fig. 1). In the Wistar rat, 70–80% of total bilirubin conjugates in bile are glucuronide conjugates; bilirubin monoglucuronide represents 80% of glucuronide conjugates, and bilirubin diglucuronide the remainder. Proportions of bilirubin conjugates in the PBS wash and in the ethanol eluate were similar to that in bile. However, 80% of glucuronide conjugates in the distilled water eluate was bilirubin diglucuronide. The Wistar-R rat, unlike other strains of Wistar rats (28), has less than 10% nonglucuronide conjugates of bilirubin; of the glucuronide conjugates, 50–60% is bilirubin diglucuronide. Chromatography of bile



l. Elution of radiolabeled bile after application to an albumin-agarose gel affinity chromatography After rat bile was applied, unbound biliary constituents were washed from the column with 0.02 *M* te buffered saline at pH 7.0 (0.02 *M* PBS). Distilled water (H_2O) eluted a fraction of pigment; 50% (50% EtOH) eluted a second fraction. The relative proportions of bilirubin mono- and diglucuronide in ›und material and the two eluates are indicated by the bars.

from a Wistar-R rat on albumin–agarose gel revealed almost exclusively bilirubin diglucuronide in the water eluate.

When tritiated bilirubin conjugates from either the water or ethanol eluates were diazotized and applied to a thin-layer plate, more than 90% of the radioactivity was recovered in the visible azo bands. Weber-Schalm fractionation (30) of these eluates, before or after lyophilization, revealed that 90% or more of the radioactivity and pigment distributed into the upper, polar layer.

The absorption spectrum of the ethanol eluate after lyophilization and subsequent solution in distilled water demonstrated a peak at 450 nm. However, there was a significant shoulder at 280 nm and marked fluorescence upon excitation at this wavelength. Because the absorption and fluorescence patterns were of the sort expected of protein and suggested contamination with albumin either from the affinity column or from the bile itself, an additional purification step was undertaken. As described above, the sample was extracted with 2 vol of chloroform to remove any possible traces of unconjugated bilirubin and subjected to gel filtration through Sephadex G-25. The spectrum of the resulting preparation demonstrated removal of the material with peak absorbance at 280 nm. This preparation did not fluoresce upon excitation at 285 nm and thin-layer chromatography with chloroform (31) again confirmed the absence of a band corresponding to unconjugated bilirubin. Ethyl anthranilate diazotization and thin-layer chromatography of this protein-free preparation revealed it to contain bilirubin diglucuronide almost exclusively, suggesting preferential binding of bilirubin diglucuronide, as compared with monoglucuronide, to the Sephadex G-25.

Glucuronic acid content was determined in two protein-free preparations of bilirubin diglucuronide as determined by thin-layer chromatography of the azo pigments. The molar ratio of glucuronic acid to bilirubin was 1.8:1 and 2.3:1, respectively.

Analysis of bile and column eluates for bilirubin and bile acids (Table I) demonstrated a marked decrease in the molar ratio of bile acids to bilirubin most evident in the ethanol and Sephadex eluates in which this ratio was 0.05:1 and <0.01:1, respectively, as compared to 11:1 in the starting bile. Simultaneous cholesterol analysis demonstrated it to be absent from all eluates; phospholipid content was 14 μmole in bile, 0.013 μmole in the water eluate, 1.7 μmole in the ethanol eluate, and 0.2 μmole in the Sephadex eluate. All phospholipid contamination was removed after extraction with chloroform–methanol; such extraction removed 10% or less of conjugated bilirubin.

When stored in a standard freezer for 2 weeks at −15°, these lyophilized preparations showed no change in Weber–Schalm fractionation. When diazotized after 2 weeks and subjected to thin-layer chromatography, less than 10% of radioactivity was lost from the visible bands. The ratio of glucuronide conjugates to other conjugates remained constant, but there was a 30 to 40% decrease in bilirubin diglucuronide content with a corresponding increase in bilirubin monoglucuronide.

Discussion. Previous attempts to purify conjugated bilirubin have resulted in preparations containing significant amounts of bile acids and other contaminants (6–19). The present method, utilizing affinity chromatography of rat bile, is relatively rapid and simple to perform, and produces a preparation of conjugated bilirubin virtually free of the major biliary contaminants. A degree of separation of monoglucuronide from diglucuronide may be accomplished by differential elution with distilled water followed by 50% ethanol. If bile from a Wistar-R rat is used, a preparation of conjugated bilirubin which is more than 90% bilirubin diglucuronide can be obtained from the water

TABLE I. PURIFICATION OF CONJUGATED BILIRUBIN FROM RAT BILE.

	Bilirubin[a]	Total bile acids[a]	Cholesterol[a]	Phospholipids[a]
Bile	2.8	31	18	14
Water eluate	0.2	0.1	0	0
Ethanol eluate	1.1	0.06	0	0
Sephadex eluate	0.2	<0.002	0	0

[a] μmole.

luate. Bilirubin diglucuronide may also be btained by subjecting the ethanol eluate to urther chromatography on Sephadex G-25. hese preparations of conjugated bilirubin re relatively stable when lyophilized and tored at −15°. Their long-term stability has et to be established.

Previous studies of hepatic organic anion ransport have been unable to directly exmine the metabolism of conjugated biliruin, in part due to difficulty in its purificaion. The method described here may faciliate investigation into this question as well s investigation into the physical characterstics and composition of the conjugates of ilirubin.

The authors would like to thank Dr. Norman Javitt or performing numerous bile acid determinations, and or constructive advice. The assistance of Dr. Robert nodell with certain additional bile acid studies is ratefully acknowledged. We would like to acknowldge Dr. Peter Herbert for advice on cholesterol deterinations, Dr. John Foulds and Dr. Tuu-Jyi Chai for erforming phospholipid determinations, and Dr. eanne Ketley for assistance with fluorimetric studies. Ve would also like to thank Dr. William Jakoby for his elpful advice and encouragement.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 24-28 (1976)

Effect of Calcium on RNA and Protein Synthesis in the Hypertrophied Myocardium (39319)

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RNA and protein synthesis are increased in the myocardium of animals subjected to experimental aortic stenosis (1-3). These processes appear to be initiated by an increase in endocardial tension; however, the mechanism by which an increase in tension is able to affect macromolecular synthesis is as yet unknown. It has been well established that the contractile process and the resultant development of tension in muscles involve Ca^{2+} flux (4-6) and that this ion also has been noted to enhance both RNA and protein synthesis (7-10). In view of these observations, it is of interest to determine if the enhancement of RNA and protein synthesis in the hypertrophied heart is related to the effect of Ca^{2+}.

Methods. Sprague-Dawley rats, weighing 220-270 g, were maintained on food and water *ad libitum*. Aortic constriction was performed with a size-19 needle (1.0-mm o.d.) by the method of Florini (2). Surgery in the sham group (control) was similar except that the thread was loosely tied around the descending aorta. Seven days after surgery the heart was excised and weighed after removal of the atria and aorta. The left ventricle was sliced and 120-150 mg of tissue was incubated in 5-ml Krebs Ringer phosphate buffer, pH 7.4, in the presence or absence of 1.5 m*M* Ca^{2+}. The incubation buffer contained 5.6 m*M* glucose, all the amino acids at normal serum concentration (11), and the radioactive precursors [U-^{14}C]leucine (0.5 μCi/ml) and [5,6-^{3}H]uridine (1.0 μCi/ml).

After the incubation period the slices were rinsed five times in the incubation buffer prior to homogenization. Proteins from the 600*g* (myofibrillar) pellet and the 105,000*g* (sarcoplasm) soluble fraction were precipitated with an equal volume of 20% trichloroacetic acid (TCA), washed, and assayed for radioactivity (14, 15) and protein concentration (12). RNA was extracted from the 600*g* fraction by an incubation for 60 min at 37° in 0.3 *M* KOH according to the method of Fleck and Munro (13), and assayed for radioactivity (15) and total RNA as described by Mejbaum (14).

Ca^{2+} levels were measured in left ventricles by digestion of 100 mg of tissue in 1:3 70% perchloric acid-30% hydrogen peroxide for 4 hr at 70°. The digestion mixture was diluted to 2.0 ml with 0.4 ml of 5% lanthanum oxide and glass double-distilled water, and assayed in Perkin Elmer atomic absorption spectrophotometer at 422.7 nm.

Results. The presence of hypertrophy was determined by assessment of the heart weight to body weight ratio which was $3.13 \pm 0.17 \times 10^{-3}$ (mean ± SD) in the hypertrophy group 7 days postsurgery as compared to $2.74 \pm 0.18 \times 10^{-3}$ (mean ± SD) in the sham ($P < 0.0025$). In further support of the development of hypertrophy is the fact that RNA concentrations were also elevated in the aortic constricted group [557 ± 54 μg/ht (mean ± SD) vs 353 ± 35 μg/ht (mean ± SD) ($P < 0.0005$)].

Although heterogenous RNA decreased after a 3-hr incubation period in both groups, in the absence of Ca^{2+} RNA was lower in the sham than in the hypertrophy group (Fig. 1). In the presence of Ca^{2+} RNA did not decrease as rapidly in the sham group, and the levels of RNA were similar to the hypertrophied group (Fig. 1).

RNA synthesis in the hypertrophy group was essentially the same as the sham (Fig. 2). Although the incorporation of [5,6-^{3}H]uridine into RNA was increased by 1.5 m*M* Ca^{2+} in both groups, no proportionate difference in the Ca^{2+} enhancement could be observed (Fig. 2). The rate of incorporation of labeled uridine into RNA in both the sham and hypertrophy group was linear and essentially the same over the entire 4-hr incubation period as seen in Fig. 2.

Protein synthesis was increased 1.5- to

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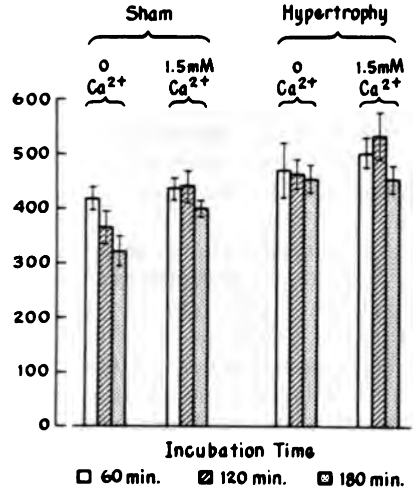

FIG. 1. A bar graph of changes in nuclear RNA concentration in slices of sham and hypertrophied hearts during a 3-hr incubation. Data are based on six experiments and expressed as total micrograms of RNA per heart ± SD.

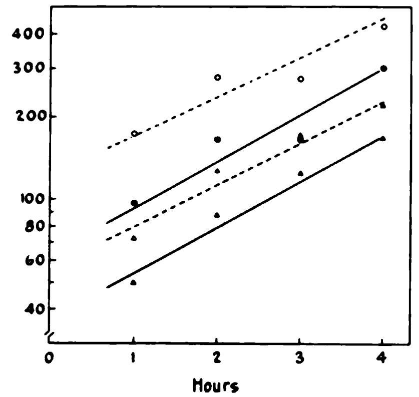

FIG. 2. A logarithmic plot of the rate of synthesis of RNA in sham- and hypertrophy-operated animals. ●——●, ○——○, sham-operated animals; ▲——▲, △——△, aortic-constricted animals; solid symbols no Ca^{2+}, open symbols 1.5 m*M* Ca^{2+}. Data are based on six experiments and are expressed as dpm/μg RNA.

2.5-fold in left ventricular slices from the hypertrophied hearts, as shown in Figs. 3–6. The addition of Ca^{2+} to the incubation buffer stimulated the rate of incorporation of [^{14}C]leucine into protein in both groups. When the incubation period was extended for 4 hr, the rate of myofibrillar-nuclear protein synthesis was decreasing after 3 hr in both the sham and hypertrophied groups (Fig. 3). A logarithmic plot of these data indicated that the rate of synthesis of protein followed first order kinetics (Fig. 4) with a 2.8 greater rate of synthesis in the hypertrophied group. During the first 2 hr Ca^{2+} stimulated more markedly the incorporation of leucine into protein in the sham group (Fig. 4).

The 105,000*g* supernatant proteins in the hypertrophy group also were labeled at a faster rate than in the sham group (Fig. 5). In constrast to the myofibrillar proteins, the soluble proteins in the sham group did not appear to slow down after a 3-hr incubation period. Both groups exhibited an exponential rate of soluble protein synthesis (Fig. 6), and the stimulation of soluble protein synthesis was initially more markedly enhanced by Ca^{2+} in the sham group.

The uptake of [^{14}C]leucine and [^{3}H]uridine into the TCA-soluble fraction over the 4-hr incubation period was not significantly different between the sham-operated and

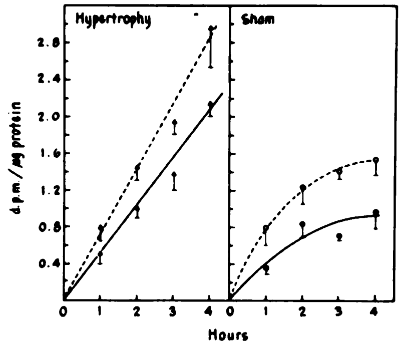

FIG. 3. The rate of incorporation of [^{14}C]leucine into nuclear myofibrillar proteins of sham- and hypertrophied-operated animals. Legend to figure is described in Fig. 2. Data are based on mean of six experiments and expressed as dpm/μg protein.

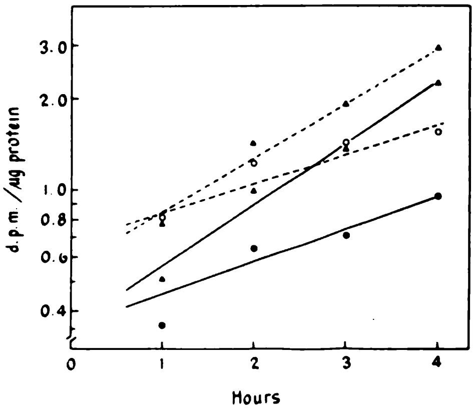

FIG. 4. A logarithmic plot of Fig. 3.

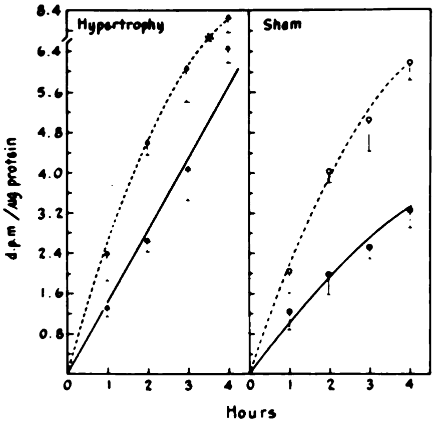

FIG. 5. The rate of incorporation of [^{14}C]leucine into soluble proteins of sham- and hypertrophied-operated animals. Legend to figure is described in Fig. 2. Data are based on mean of six experiments and expressed as dpm/µg protein.

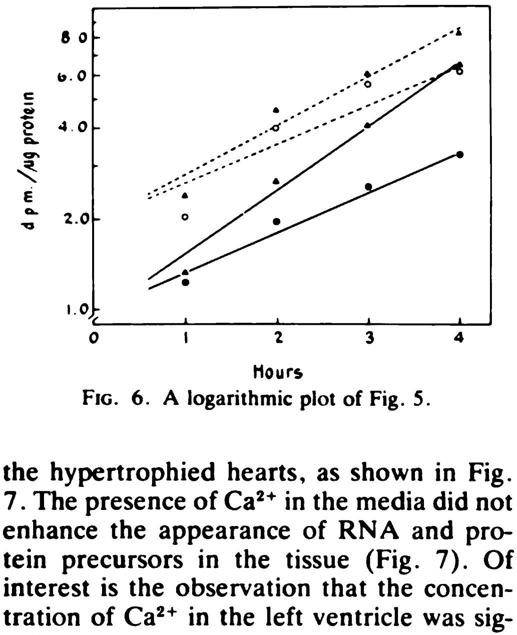

FIG. 6. A logarithmic plot of Fig. 5.

the hypertrophied hearts, as shown in Fig. 7. The presence of Ca^{2+} in the media did not enhance the appearance of RNA and protein precursors in the tissue (Fig. 7). Of interest is the observation that the concentration of Ca^{2+} in the left ventricle was significantly higher 4.8 ± 0.4 mg of Ca^{2+}/100 g wet wt (mean ± SD) in the nonincubated tissue of the hypertrophied hearts compared to 3.0 ± 0.6 mg Ca^{2+}/100 g wet wt (mean ± SD) in the sham hearts ($P < 0.0005$).

Discussion. These studies indicate that the rate of synthesis of RNA and of proteins in myocardial slices from rats subjected to 7 days of experimental aortic outflow obstruction are similar to *in vivo* experiments (1–3). The data demonstrate an enhanced rate of protein but not RNA synthesis at this time interval.

It was noted that protein synthesis in both the sham and hypertrophied groups was stimulated by the presence of exogenous calcium. However, the rate of soluble protein synthesis in the hypertrophied group was proportionately no greater than in the sham. Our observations, as well as others (15–18), indicate that endogenous calcium is elevated in hypertrophy hearts. It is possible that the elevation of intracellular Ca^{2+} may be responsible for the increased incorporation of labeled amino acid into protein (19) in the hypertrophied myocardium.

Another mechanism which may enhance protein synthesis is an increase in concentration of RNA (1, 20). This RNA may also have a long half-life since protein synthesis was not diminished in the hypertrophied group after 7 days. The elevated levels of RNA in the hypertrophied group may be also due to an initial increased rate of synthesis of RNA (1, 20) or decreased degradation of RNA. The initial increased rate of synthesis of RNA may have been stimulated by an increased level of endogenous Ca^{2+} (8, 19) during the early development of hypertrophy.

The results of this study also indicate that *in vitro* the level of RNA in the hypertrophy group did not decrease as rapidly as the sham (Fig. 1), and this decrease can be replicated in the sham animals by the presence of Ca^{2+}. Calcium has been reported to prevent the breakdown of lysosomes (21.

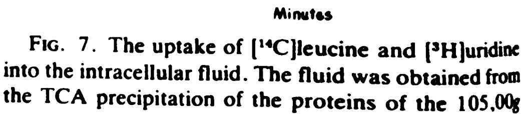

FIG. 7. The uptake of [^{14}C]leucine and [^{3}H]uridine into the intracellular fluid. The fluid was obtained from the TCA precipitation of the proteins of the 105,00g soluble supernatant solution. See legend to Fig. 2 for description of symbols.

2) which contain the enzyme involved in RNA degradation, RNase (23). Since Ca^{2+} evels are higher in the hypertrophied heart, he higher concentration of RNA in this issue may be due to a direct inhibition of RNase, to an inhibition of its release from he lysosomes or decreased leakage out of he cell (21, 22). This may be of importance o the enhanced rate of protein synthesis since total RNA levels in the hypertrophied group remained higher for 2-3 weeks following aortic banding (1) whereas RNA synthesis was elevated only in the early stage of hypertrophy (1, 20).

Although tissue slice preparations have rregular, damaged cells, both RNA and protein are synthesized at a linear rate for 2-3 hr (7, 8, 19), suggesting that the cells are viable. In addition, no differences in the uptake of uridine and leucine were observed between the hypertrophy and sham groups (Fig. 7) using the tissue preparation. It would appear that the differences in the rate of RNA and protein metabolism are not due to a change in the rate of uptake of uridine or leucine into the sarcoplasmic pool from the extracellular media (8-10, 24-28). However, it is conceivable that the precursors may be transported more rapidly into an intracellular pool to be utilized faster in the hypertrophied heart, or that there may be small intracellular pools which have different intracellular concentrations of the precursors.

It can be observed that extracellular calcium enhances RNA and protein synthesis in both groups; but a more marked stimulation of the rate of sarcoplasmic protein synthesis by calcium was noted only in the sham group. The increased rate of basal protein synthesis in the hypertrophied group may be related to (i) increased levels of intracellular calcium or (ii) an increase in RNA concentration. In either case an increase in Ca^{2+} flux may be a significant determinant in the process associated with myocardial hypertrophy.

Summary. Myocardial protein synthesis was elevated 7 days after rats were subjected to experimental aortic outflow obstruction. Although RNA synthesis was not increased at this time, RNA concentration was elevated and may have provided for the observed increase in protein synthesis. A possible basis for the persistence of the high RNA levels was a decrease in the degradation of RNA. The increase in intracellular calcium observed in hypertrophied tissue may be involved in the maintenance of RNA concentration and in the increased rate of protein synthesis.

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OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 29–32 (1976)

ression of Corpora Lutea in the Rabbit after Injection of a Gonadotropin-Releasing Peptide (39320)

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(Introduced by E. T. KIMURA)

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al function of corpora lutea is re- throughout pregnancy in the rabbit re adequate synthesis and secretion esterone. When large doses of exog- uteinizing hormone (LH) were given dopregnant rabbits (1), ovulation of- urred followed by regression of func- orpora lutea. Subsequently, it was that estrogen prevented LH-induced ion of corpora lutea (2, 3) and this is now accepted as the ultimate lu- ic hormone in the rabbit, necessary ntenance of corpora lutea once they rmed. Recent experiments (4) indi- at the luteotropic stimulus of estro- ches the corpus luteum by way of the blood supply.

importance of the ovarian follicle as rce of 17β-estradiol was demon- when the follicles were destroyed by iation and the corpora lutea re- and no longer synthesized or se- rogesterone (5). Moreover, daily in- of estradiol prevented luteolysis by the destruction of ovarian folli- hus, it was proposed that the luteo- tivity of exogenous LH was second- reovulation which removed the of endogenous estrogen (6).

s the purpose of this study to deter- he influence of [D-Leu^6, des-Gly Pro-ethylamide^9]-GnRH, and LH- g peptide (7, 8), on corpora lutea n in both pregnant and pseudopreg- bbits.

rials and methods. Details of the pro- for the synthesis, deblocking, and tion of synthetic [D-Leu^6, des-Gly Pro-ethylamide^9]-GnRH were de- previously (7).

re New Zealand does (3.5 to 4.5 kg) ged individually in a temperature- ntrolled room (25°; lights on 6 AM–8 at least a 3-week period before experimentation. Pseudopregnancy was induced in 12 does by an iv injection of 25 I.U. HCG[1]. All others does were force-mated within a 2-hr period to males of proven fertility and randomly assigned to treatment. They also received an injection of HCG within 5 min of the initial mating. The corpora lutea of pseudopregnancy and pregnancy were marked with India ink on Day 2 to distinguish them from peptide-induced corpora lutea. The peptide was diluted in 0.1% bovine serum albumin and injected into a marginal ear vein. Sesame oil was used as diluent for 17β-estradiol[2] and progesterone, which was administered subcutaneously.

Blood was collected from an ear vein and immediately refrigerated. Within 24 hr the blood samples were centrifuged (2500 rpm) and the serum stored at −20°. Progesterone was measured by a competitive protein-binding assay (9). The rabbits were killed by injecting an air embolism into an ear vein after which the entire reproductive tract was removed and examined for viable and non-viable fetuses. Corpora lutea were removed from the ovaries, cleaned of adhering tissue, and weighed on a torsion balance to the nearest 0.2 mg. The data were subjected to analysis of variance and differences among treatments determined by Duncan's multiple range test (10).

Results. Injection of 50 μg of the peptide on Day 11 of pseudopregnancy reduced ($P < 0.01$) the weight of functioning corpora lutea by Day 17 (16.3 vs 5.1 mg) and induced formation of additional corpora lutea (Table I). Estradiol maintained the weight of corpora lutea of pseudopregnancy

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[1] Antuitrin "S," Parke-Davis, Ann Arbor, Michigan.

[2] Progynon suspension, Schering Corp., Bloomfield, New Jersey.

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of peptide-treated rabbits, but did not affect the weight of the "new" corpora lutea.

A single 50-μg injection of the peptide on either Day 10, 14, or 19 of pregnancy resulted in 34, 84, and 83% fetal resorptions, respectively, compared to 12% in controls, as observed on Day 28. Four rabbits were examined in each group. The presence of two distinct sets of corpora lutea on the ovaries of peptide-treated rabbits suggested that sufficient LH was released from the pituitary to induce ovulation or cause luteinization of existing follicles. Moreover, there was no affect on percentage of fetal resorption in rabbits which did not ovulate after receiving lower dose levels of either 2 or 10 μg of the peptide.

In another experiment, 50 μg of the peptide injected on Day 15 of pregnancy induced formation of corpora lutea and regression of the functioning corpora lutea (Table II). Serum progesterone was reduced ($P < 0.01$) within 3 days of peptide injection but increased again by Day 23, undoubtedly reflecting progesterone secretion by the "new" corpora lutea (Fig. 1). The decline in serum progesterone in controls by Day 23 is consistent with the decline in ovarian production (11) and circulating levels of progesterone (12) during the last third of pregnancy. Fetal resorption determined at Day 23 was 85 and 4.1%, respectively, for peptide-treated and control animals (Table II). Twice a day injection of 31.2 and 125 ng of estradiol into peptide-treated rabbits did not prevent corpora lutea weight loss or fetal resorption. However, at 500 ng, estradiol prevented corpora lutea regression and fetal loss induced by the peptide, resulting in values similar to controls. In contrast, a dose of 2 μg of estradiol twice daily maintained corpora lutea weight in peptide-treated does, but had a negative effect on fetal survival. Progesterone (25 mg, twice daily) maintained both corpora lutea weight and fetal survival at levels comparable to controls.

Discussion. These experiments demon-

TABLE I. EFFECT OF [D-Leu^6, des-Gly NH$_2$^{10}, PRO-ETHYLAMIDE^9]-GnRH AND ESTRADIOL ON CORPORA LUTEA WEIGHT IN THE PSEUDOPREGNANT RABBIT.

	Corpora lutea of pseudopregnancy		Peptide-induced corpora lutea	
Group[a]	Number	Weight (mg ± SE)	Number	Weight (mg ± SE)
1. Controls	16.0	16.3 ± 1.1	–	–
2. Peptide (P)[b]	11.2	5.1 ± 0.4	12.8	8.2 ± 0.3
3. P + Estradiol[c]	12.8	18.3 ± 2.4	9.5	11.2 ± 0.8

[a] Four animals per group.

[b] Single injection of 50 μg on Day 11.

[c] Twice daily injection of 1 μg of 17β-estradiol beginning on Day 11. All rabbits were autopsied on Day 17.

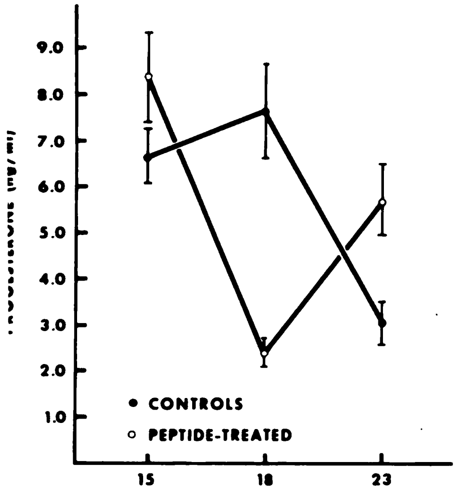
TABLE II. EFFECT OF ESTRADIOL (E_2) AND PROGESTERONE (P_4) ON CORPORA LUTEA WEIGHT AND FETAL SURVIVAL IN [D-Leu^6, des-Gly NH$_2$^{10}, Pro-Ethylamide^9]-GnRH-TREATED PREGNANT RABBITS.

	Number of does implanting per number mated	Mean number implants	Mean number viable fetuses	Resorptions (%)	Mean number corpora lutea		Mean corpora lutea weight (mg)	
					Pregnancy	Induced	Pregnancy	Induced
Controls	11/12	9.00 ± 0.73[c]	8.63 ± 0.64	4.1	11.10 ± 0.47	–	20.40 ± 1.30	–
Peptide (P)[a]	12/13	9.50 ± 0.58	1.42 ± 0.60	85	9.53 ± 0.79	14.2 ± 0.86	7.63 ± 1.20	13.2 ± 1.00
P + E_2[b] (31.2 ng)	5/5	6.00 ± 1.10	0.42 ± 0.24	93	7.80 ± 0.43	12.4 ± 0.66	8.10 ± 0.98	14.3 ± 0.36
P + E_2 (125 ng)	4/5	9.00 ± 1.20	2.00 ± 1.20	78	11.80 ± 0.89	11.0 ± 1.83	7.60 ± 2.40	11.3 ± 2.60
P + E_2 (500 ng)	5/5	7.60 ± 0.51	7.00 ± 0.71	7.9	11.60 ± 1.00	8.8 ± 1.30	16.60 ± 1.40	16.4 ± 0.98
P + E_2 (2000 ng)	5/5	10.00 ± 0.89	0.40 ± 0.24	96	10.40 ± 0.85	11.4 ± 0.82	16.70 ± 0.67	14.2 ± 0.41
P + P_4 (25 mg)	7/8	7.30 ± 1.20	7.00 ± 1.30	4.1	8.70 ± 0.80	10.7 ± 1.00	15.80 ± 1.50	16.5 ± 1.20

[a] Single iv injection of 50 μg on Day 15.

[b] Twice-daily injections of E_2 and P_4 sc in sesame oil.

[c] SE of mean



IG. 1. Serum progesterone concentrations in nant rabbits before and after the injection of vehi- or [D-Leu^6, des-Gly NH$_2$^{10}, Pro-ethylamide^9]- ιH. Each point represents the mean ± SE of eight ..

te that a potent gonadotropin-releasing tide apparently induces sufficient LH ase from the pituitary of the pseudo- gnant and pregnant rabbit to cause ovu- n and regression of existing corpora lu-

That the peptide induced ovulation in pregnant rabbit is supported by recent eriments (unpublished) in which we ob- ed ovulation points on the ovary and vered eggs from the oviducts 24 hr after ction. To our knowledge, this is the first ionstration that endogenously released will ovulate the estrogen-secreting folli- present during pseudopregnancy and nancy, thus depriving the rabbit of its otropic support for existing corpora lu- In pregnant rabbits, serum progester- declined within 3 days of peptide injec- . Furthermore, corpora lutea regression lted in a large percentage of fetal re- tions. In many of the rabbits, complete tion occurred when the peptide was n on Day 19 of pregnancy.

hat the luteolytic effect of the peptide is ndent on ovulation is supported by the rvations that certain levels of estradiol maintain corpora lutea weight and fetal ility. Keyes and Nalbandov (5) reported 2 to 4 μg of estradiol was required daily aintain function of corpora lutea in rab- in which the ovaries had been X-irradiated. However, we have found that injecting 0.5 μg of the steroid twice daily maintained both corpora lutea weight and fetal survival. These differences are probably related to the method of destruction of estrogen-secreting ovarian tissue, in that X-irradiation is undoubtedly a more severe treatment than peptide-induced ovulation. In our studies the twice-a-day dose of 2 μg of estradiol maintained corpora lutea weight but was detrimental to fetal survival. The fact that peptide-induced luteolysis was prevented by progesterone is surprising in view of the accepted luteotropic property assigned to estrogen. Possibly the large dose of progesterone protected or sensitized the estradiol receptors of the corpora lutea to the existing low levels of that steroid or allowed for sufficient conversion to estrogen. Verification of these possibilities awaits further experimentation.

Summary. We have shown that a potent agonist of the luteinizing hormone injected on either Day 11 of pseudopregnancy or Day 15 of pregnancy causes luteinization of ovarian follicles and a rapid regression of functional corpora lutea. Serum levels of progesterone declined markedly within 3 days of peptide injection in pregnant animals. In the presence of regressing corpora lutea, fetal resorption occurs which can be prevented by estradiol (500 ng, twice a day) or progesterone. Daily levels of estradiol below 1 μg did not maintain either corpora lutea weight or fetal survival, whereas 4 μg of the steroid maintained corpora lutea weight, but not pregnancy.

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ired Thymic Regeneration in Lethally Irradiated Mice Given Bone Marrow from Aged Donors[1]

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humoral and cell-mediated immune es decline with advancing age as the f complex changes in the immune and its environment (1). In part this can be attributed to a decrease in the ative capacity of mature T-cells. This ion is based on observations demon- that although the number of θ posi- ls in mouse lymphoid tissues remains ly constant (1), the proliferative re- of these cells to T-cell mitogens (2), eic cells (3), and to thymus-depend- tigens (4) decrease markedly with

te, studies on age-related changes in immunity have focused on the func- mature post-thymic T-cells obtained ly from peripheral lymphoid tissues, le or no information has been devel- the effects of aging on the precur- these cells. Reported here are obser- suggesting that with age there is an e decrease in bone marrow T-cell tors and/or an impairment of their ative capacity.

ials and methods. Mice. Male /6 × A/HeJ)F_1, B6AF_1, mice were ed from The Jackson Laboratory, rbor, Maine. Bone marrow recipi- re 12- to 16-weeks old; they were 00 R of whole-body γ radiation on of cell transfer (^{60}Co; 100 R/min; o-source, 80 cm; 100% lethal to un- d controls in 11–14 days). After ir- n the mice were housed five per d for 21 days they were given water ing neomycin. Bone marrow donors to 143-week-old B6AF_1 males.

marrow. Donors were killed by cer- slocation. Bone marrow cells were from the femoral cavity with Hank's , and they were washed twice. The ed cells were counted in triplicate in a hemocytometer. Cell viability was assayed by the trypan blue exclusion test, and in all cases 90 to 93% of the cells excluded the dye. After appropriate adjustments in cell concentration, the cells were injected iv in 0.2 ml into the lateral tail vein of irradiated recipients.

Regeneration of thymus and spleen. It is generally accepted that only T-cell precursors repopulate the thymus of lethally irradiated, bone marrow-reconstituted recipients (5). Therefore, irradiated mice were injected iv with 2.8 to 3.2 × 10^6 viable nucleated marrow cells obtained from 10- to 143-week-old syngeneic donors, and 3 weeks later their spleens and thymuses were removed and weighed individually. This time period was selected because preliminary work showed that repopulation of the thymus by donor cells was nearly maximal by 21 days after injection and host marrow recovery, if any, was minimal at this time (unpublished data). The results of these experiments have been expressed in terms of milligrams of organ weight per 10^6 cells injected. The data have been analyzed by means of Student's *t* test.

Colony-forming units (CFU) assay (6). Previous studies have shown that with most strains of mice there is little or no change with age in the number of hematopoietic stem cells (CFU) in the marrow (7, 8). Therefore, as a check on the reliability of the total cell counts and cell viability determinations, irradiated mice were injected iv with 1 to 1.5 × 10^5 marrow cells from the preparations used to measure lymphoid organ regeneration. Nine days later the mice were killed, and their spleens were fixed in Bouin's solution. CFU were counted under 4× magnification.

Results and discussion. Although the number of CFU varied somewhat between experiments, total CFU per femur and per 10^5 cells remained relatively constant between 14 and 127 weeks (Table I). These

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TABLE I. SPLEEN CFU AND THYMUS AND SPLEEN WEIGHTS OF LETHALLY IRRADIATED B6AF$_1$ MICE GIVEN MARROW CELLS FROM AGING SYNGENEIC DONORS.[a]

	CFU			Organ weight (mg ± SD/10^6 cell)		
Age of donor (weeks)	Number of mice	Per femur	Per 10^5 cells	Number of mice	Thymus	Spleen
16	5	1545	13.5	7	9.9 ± 1.1	21.6 ± 3.6
63	5	1512	14.6	9	10.1 ± 0.9	19.2 ± 2.5
16	5	1612	11.5	5	9.8 ± 1.2	16.0 ± 2.7
71	5	1555	12.7	5	8.9 ± 1.1	16.1 ± 3.1
10				4	10.4 ± 0.8	21.6 ± 2.2
117				5	7.1 ± 0.7[b]	17.7 ± 2.6
14	6	2220	15.7	5	10.1 ± 1.2	23.7 ± 1.8
122	5	1884	14.8	5	6.8 ± 1.1[b]	24.1 ± 3.0
14	5	1643	11.3	5	10.3 ± 0.7	23.6 ± 2.4
124	5	1795	13.6	5	6.4 ± 0.9[b]	20.1 ± 1.8
16	5	2209	13.7	5	9.8 ± 0.9	23.3 ± 1.9
127	5	2034	14.5	5	5.2 ± 0.7[b]	23.6 ± 2.1
16				5	9.4 ± 0.9	24.6 ± 2.4
143				5	4.3 ± 0.3[b]	16.8 ± 2.6[b]

[a] The mice were exposed to 900 R ^{60}Co γ-radiation and given 1.0–1.5 × 10^5 or 2.8–3.2 × 10^6 marrow cells iv. Nine days later CFU were counted in the spleens of mice given 10^5 cells, and 21 days after irradiation, spleen and thymus weights were determined in recipients of 10^6 cells.

[b] $P < 0.01$, Student's *t* test.

findings are consistent with previous work which demonstrated that in healthy mice total marrow cellularity and the hematopoietic stem cell population were little affected by aging (7, 8).

Similarly, the regeneration of spleen weight was not consistently or significantly affected by the age of the marrow donor. The one exception to this was found in the group given marrow from 143-week-old donors; here splenic regeneration was significantly diminished. However, the reproducibility and the biological significance of this finding remain to be determined.

By contrast, thymic regeneration was significantly impaired when the irradiated recipients were given marrow cells from donors 117 weeks of age or older; further, there appeared to be an inverse relationship between the age of the donor and the ability of the marrow to repopulate the thymus.

Although it has been shown that marrow from aged donors has a diminished ability to repopulate the thymus of irradiated hosts, a function reserved to precursors of T-cells, it is not clear whether this is the result of a decrease in the size of the stem cell pool and/or an impairment of proliferative capacity. The resolution of these questions may await the discovery of a unique cell marker for the precursors of T-cells. In experiments not reported here, it has been found that although the graft-vs-host activity of spleen cells from 126-week-old mice was significantly diminished, 10 × 10^6 marrow cells from these aged donors produced the same mortality among irradiated recipients (4/20) as did equal numbers of cells from 12-week-old mice. This suggests that the numbers of post-thymic effector T-cells in the marrow inoculae were not grossly different.

Summary. Mice were given a lethal dose of whole-body γ radiation and injected with 10^5 or 10^6 marrow cells from 10- to 143-week-old syngeneic donors. Nine days later, colony-forming units (CFU) were counted in the spleens of mice given 10^5 cells, and 21 days after irradiation, spleen and thymus weights were determined in the recipients of 10^6 cells. It was found that there were no significant changes with age in marrow CFU or in the ability of marrow cells to repopulate host spleens. In contrast, thymic regeneration was significantly impaired when the recipients received marrow cells from donors 117 weeks of age or older. These observations suggest that with aging there is a decrease in marrow T-cell progenitors and/or a decline in their proliferative capacity.

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Variations in Elastaselike Esterase Activities in Human Leucocytes during Cell Maturation[1] (39322)

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The involvement of leucocyte enzymes in the pathogenesis of inflammatory diseases has been the subject of several recent reviews and symposia (1–4). Human leucocytes contain several different neutral esterases and proteases (5, 6), most of which are located in the azurophil granules of these cells (6). One of these enzymes, an elastaselike esterase which migrates slowly in acrylamide gel during acid-pH electrophoresis, requires the presence of Triton to hydrolyze synthetic substrates (5). In this regard, the slow-migrating enzyme is different from the well-characterized, rapidly migrating elastase isozymes present in the same leucocyte granules (7–10), since the latter enzymes are capable of hydrolyzing substrates in the absence of the detergent. In the course of recent work on the purification of leucocyte elastases (10), we observed that granular extracts from preleukemic and leukemic individuals contained the slow-moving, Triton-activatable elastaselike esterase (SE), but no elastase isozymes. One possibility that was suggested by this observation was that SE might be an inactive zymogen or precursor form of leucocyte elastase found mainly in immature cells, but partly retained as the cells matured.

The purpose of the present studies was to explore the relationship between cellular maturation and the SE content of human leucocyte granules. We also studied some of the biochemical and immunological properties of leucocyte SE.

Materials. Human blood from healthy individuals was purchased from the N.Y. Blood Center. Bone marrow aspirates from nonleukemic patients were kindly provided by Dr. R. Prasad of the V.A. Hospital, Northport, N.Y. Leucocytes from both sources were processed within a few hours. Enzyme substrates and inhibitors were the same as reported earlier (10). Rabbit antiserum to human leucocyte elastase was prepared in this laboratory as described before (10), and GARG[4] was purchased from Meloy Laboratories, Inc., Springfield, Virginia. The latter antiserum contained 9.0 mg/ml of antibody protein. Elastatinal was a gift from Dr. T. Sugimura.

Methods. Human leucocytes and leucocyte granules were isolated from venous blood and bone marrow aspirates by the procedure of Janoff and Scherer (11). Lysis of the granules in neutral-buffered 0.10 *M* NaCl to provide a crude extract of granule proteins was carried out as described elsewhere (10). Acrylamide gel electrophoresis, at pH 4.3, was performed according to Reisfeld *et al.* (12). Protein concentrations were determined by the method of Lowry *et al.* (13) using bovine serum albumin as a standard. Enzymatic staining reactions in acrylamide gels using Ac-DL-Ala-1-ONap was done according to Sweetman and Ornstein (5) with slight modifications (14). Enzymatic assays for elastase using Boc-Ala-ONp as a substrate were carried out as described before (10).

A double immunodiffusion experiment to test for antigenic cross-reactivity between SE and the rapidly-migrating leucocyte elas-

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[4] Abbreviations: *N*-acetyl-L-alanyl-L-alanyl-L-prolyl-L-alanine chloromethyl ketone, Ac-(Ala)$_2$-Pro-AlaCH$_2$Cl; *N*-acetyl-L-alanyl-L-alanyl-L-alanine chloromethyl ketone, Ac-(Ala)$_3$CH$_2$Cl; carbobenzoxy-L-alanine chloromethyl ketone, Z-AlaCH$_2$Cl; *N*-*t*-butyloxycarbonyl-L-alanine-*p*-nitrophenol, Boc-Ala-ONp; *N*-acetyl-DL-alanine-α-naphthyl ester, Ac-DL-Ala-1-ONap; Diisopropylfluorophosphate, Dip-F; Goat anti-rabbit immunoglobulins antiserum, GARG; slow-moving esterases, SE.

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ıozymes was performed as follows. undred-sixty-seven micrograms of e extract protein were loaded onto ıf four acrylamide gels, and standard ic disk-electrophoresis was carried out Ⅵfter electrophoresis, the anodal seg- ɔf each gel was removed. These seg- contained the SE proteins, as judged ɔther gels stained for protein content r esterase activity against Ac-DL-Ala- ıp. Care was taken to exclude por- ɔf the gel containing the rapidly mi- ; elastase isozymes. The anodal seg- were then lyophilized, pooled, pul- ɟ with a glass rod, and extracted in 5.0 ıCl + 0.05 *M* sodium phosphate , pH 7.5, for 6 hr in the cold. The t was clarified by centrifugation, con- ted by ultrafiltration (Amicon XM-50 rane), dialyzed vs H_2O, and lyophi- The lyophilate was restored to 0.1-ml th H_2O. Forty microliters were sub- to cationic electrophoresis in each of w acrylamide gels, which were subse- y stained either for protein or for es- activity vs Ac-DL-Ala-1-ONap. This ɔne to check the original gel extract :overy of active SE as well as for the ce of contaminating elastase iso- . [Previous work (14) has shown that : as 0.7 μg of elastase isozymes can be ɜd in gels stained with Ac-DL-Ala-1- .] The remainder of the reconstituted :ate (20 μl) was used to fill the well of ıunodiffusion agarose plate. An adja- 'ell was filled with 3 μg of purified yte elastase isozymes (10), and a third ceived 20 μl of Amicon filtrate. The well of the plate was filled with a pecific rabbit antiserum prepared t purified human leucocyte elastase ıes (10). Precipitin lines which subse- y developed were amplified by sec- ʳ immunodiffusion of the exhaustively ɟ agarose plate against goat anti-rab- nunoglobulin antiserum (GARG).

ılts and discussion. Typical electro- ic gel patterns of human blood leuco- ·anular extracts are shown in Fig. 1. Ⅴ was stained for proteins and shows le bands. The four leucocyte elastase es are in the middle third of the gel e marked with single arrows in Fig. ɘl 1B was stained with Ac-DL-Ala-1-ONap. The elastase isozymes are revealed in the midportion of the gel as zones of reaction product, while the anodal part of the gel (top) contains an additional zone of similar esterase activity. This is the slow-moving esterase (SE).

The esterase activity of SE required the presence of Triton in the gel (0.4%) whereas that of the more rapidly moving isozymes did not, an observation previously reported by Sweetman and Ornstein (5) and confirmed by one of us (7). These results are shown in Fig. 1C, which represents a disk gel stained for esterase activity in the absence of Triton.

The slow rate of migration of SE relative to the elastase isozymes can be seen in Fig.

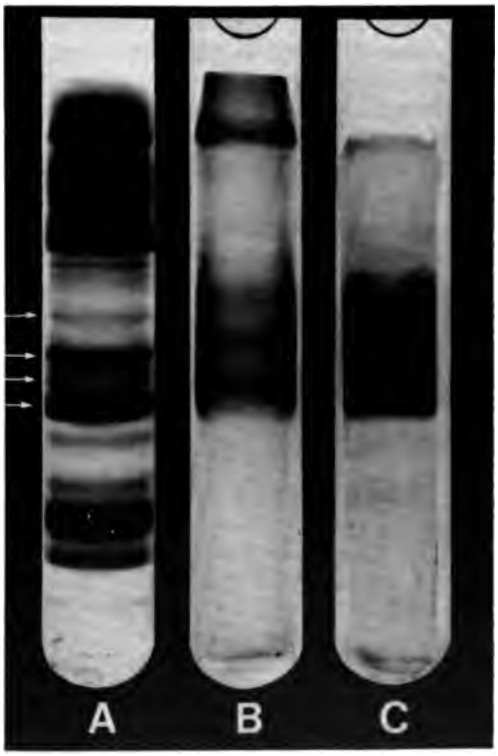


FIG. 1. Acrylamide electrophoretic gel patterns of human blood leucocyte granule extracts. Migration from top to bottom. Each gel was loaded with 535 μg of granular proteins. (A) Buffalo Black stain; (B) Ac-DL-Ala-1-ONap stain; (C) same as (B), but without Triton in the acrylamide gel. Arrows in gel (A) mark the location of four elastase isozymes. (Note that although gel (C) is shorter than gels (A) and (B), its top corresponds to the interface between spacer and separation gels as do the tops of the other two gels shown in the figure.)

1. Quantitative determination of esterase activity in gels stained with Ac-DL-Ala-1-ONap (14) showed that 10–15% of the total granule activity against this substrate was due to SE in the anodal region.

The effect of Triton on the enzymatic activity of SE against Boc-Ala-ONp was also tested as follows. After electrophoresis of leucocyte granular extract, the anodal parts (3–4 mm) of the gels were cut off and lyophilized. The lyophilized gel was extracted with neutral, 0.1 *M* phosphate buffer, and the extracts were concentrated and lyophilized again. Boc-Ala-ONp esterase activity of the final redissolved lyophilate was then determined. When 0.4% Triton was included in the enzymatic reaction mixture, the esterase activity was 61% greater than in the case of reaction mixtures not containing Triton. Detergents like Triton may affect either the conformation or state of aggregation of SE allowing it to become active against Ac-DL-Ala-1-ONap and to increase its activity against Boc-Ala-ONp, both typical leucocyte elastase substrates.

Several additional experiments were carried out to further characterize the activation and inhibition properties of SE. Treatment of leucocyte granule extract with 6.6 × 10^{-4} *M* Dip-F prior to electrophoresis resulted in complete inhibition of the esterase activity of elastases as well as of SE (Fig. 2B). Preincubation with 6.6 × 10^{-4} *M* Ac-$(Ala)_2$-Pro-AlaCH$_2$Cl, a powerful elastase inactivator (15), completely inactivated the elastase isozymes with no effect on SE (Fig. 2C). The inability of this elastase inactivator to affect SE was observed, even though Triton was present during the preincubation. Sweetman and Ornstein (5) reported that Ac-$(Ala)_3CH_2Cl$, in low concentration, did not affect SE while it did inactivate the elastase isozymes. When they increased the inactivator concentration to 2 × 10^{-3} *M*, they achieved appreciable inactivation of SE as well. In our experience, neither 6.6 × 10^{-4} *M* Ac-$(Ala)_2$-Pro-AlaCH$_2$Cl nor 6.6 × 10^{-4} *M* Z-AlaCH$_2$Cl was able to affect SE in gels. However, we did not test 10^{-3} *M* concentrations of these inactivators. Preincubation of granule extract with elastatinal, a powerful microbial peptide inhibitor of pancreatic elastase (16), did not affect the esterase activities in acrylamide gels of either the elastase isozymes or SE. This result was consonant with earlier observations of the present authors, namely that elastatinal is a rather weak inhibitor of human leucocyte

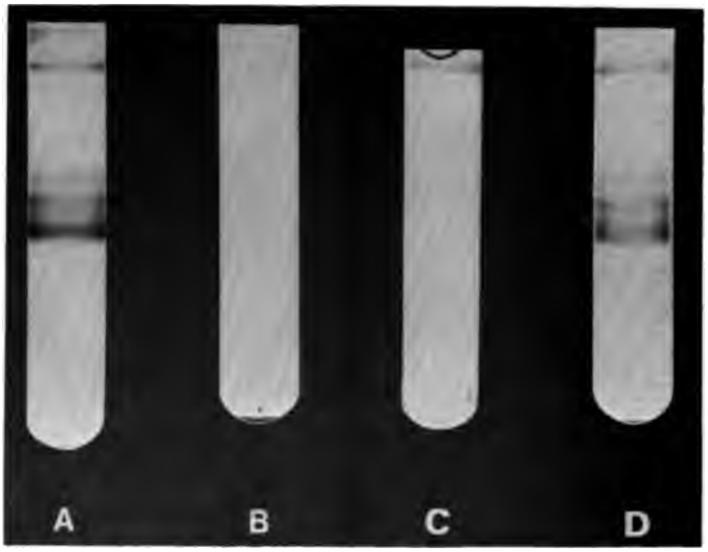
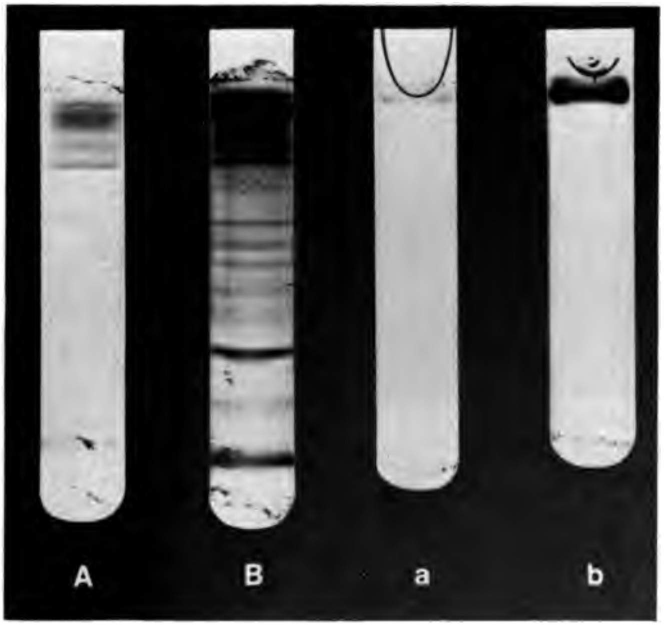


FIG. 2. Acrylamide electrophoretic gel patterns of human blood leucocyte extracts pretreated with inhibitors. Migration from top to bottom. Each gel was loaded with 107 μg of granular proteins. The granular extracts, before being loaded onto the gels, were preincubated for 1 hr at pH 7.5, at room temperature, with various inhibitors. All the gels were stained with Ac-DL-Ala-1-ONap. (A) Control (incubated without inhibitor); (B) preincubated with 6.6 × 10^{-4} *M* Dip-F; (C) preincubated with 6.6 × 10^{-4} *M* Ac-$(Ala)_2$-Pro-AlaCH$_2$Cl; (D) preincubated with 400 μg of elastatinal.

se, in comparison to porcine elastase

ylamide gel electrophoretic patterns *M* NaCl granule extracts from two rations of human bone marrow leuco- are shown in Fig. 3. As can be seen, el electrophoretic patterns (protein) of bone marrow leucocyte extracts d to be significantly different from of peripheral blood leucocytes (com- Fig. 1A to Fig. 3A and B). From the n staining, it appeared that the con- of elastase isozymes (middle region) uch lower in bone marrow leucocyte le extracts than in granule extracts peripheral blood leucocytes. This dif- e was even more strikingly demon- d when the esterase activities against -Ala-1-ONap were compared (see B vs Fig. 3a and b). No trace of elas- sozyme activities (middle region) ap- d in the gels containing bone marrow yte granule extracts. The only visible se activity against Ac-DL-Ala-1-ONap ed in the anodal portions of the gels. ugh the esterase stains shown in Fig. d C (blood leucocyte granules) were ed with large quantities of granular protein (535 μg), we previously showed that the Ac-DL-Ala-1-ONap gel-staining method is sensitive enough to detect 0.7 μg of pure leucocyte elastase isoenzymes (14) and that this method easily demonstrates these enzymes in as little as 10 μg of crude granular protein derived from blood leukocytes (7). Therefore, the total absence of isoenzyme staining in Fig. 3 (gels a and b), in which 94 and 200 μg of marrow leucocyte granular protein were tested, clearly suggests that enzymatically mature elastase isoenzymes are deficient in marrow leucocytes. The differential leucocyte count of these two bone marrow samples is given in Table I. Morphologically mature leucocytes made up about 50% of the total. Thus, despite the presence of a significant number of morphologically mature cells, enzymatic maturity was apparently still lacking, at least in the case of the elastase isozymes.

For a final experiment, SE was separated from the elastase isozymes by preparative gel electrophoresis of granule extracts prepared from peripheral blood leucocytes. Various fractions extracted from the preparative acrylamide gels (as described in *Methods*) were then tested by double immunodif-



. Acrylamide electrophoretic gel patterns of human bone marrow leucocyte granule extracts. Migration to bottom. Gels (A) and (a) were each loaded with 94 μg of granular proteins from bone marrow No. 1. and (b) were each loaded with 200 μg of granular proteins from bone marrow No. 2. Gels (A) and (B), Black stain; gels (a) and (b), reacted with Ac-DL-Ala-1-ONap for esterase activity.

fusion against a monospecific rabbit antiserum to purified leucocyte elastase isozymes (10). Evidence of antigenic cross-reactivity between the isozymes and SE was obtained (see Fig. 4). A precipitin line (single arrow) developed between the antiserum well and the well containing SE, and the line was continuous with the precipitin line (double arrow) given by pure leucocyte elastase isozymes reacting against this same antiserum. Electrophoretic analysis of the SE fractions used in this experiment revealed active SE, as expected, but failed to detect any contamination of the fractions with elastase isozymes.

Overall, the foregoing studies suggest several conclusions. The first is that during the maturation process of leucocytes, there are not only morphological changes in the cells but also biochemical-functional changes in their enzymatic contents. Elastase isozymes are stored in the azurophil granules of neutrophil leucocytes (6) and these azurophil granules appear, morphologically, in the promyelocyte stage of development (18). Yet, in our experience, active elastase isozymes could not be demonstrated functionally in marrow leucocytes, the preponderant majority of which were beyond the promyelocyte stage of their maturation process (see Table I). Morphologically mature leucocytes made up about 50% of the total. Thus, despite the presence of a significant number of morphologically mature cells, enzymatic maturity was apparently still lacking, at least in the case of the elastase isozymes. We had previously noticed that extracts of preleukemic and leukemic leucocyte granules were also deficient in elastase isozymes, but did contain SE activity.

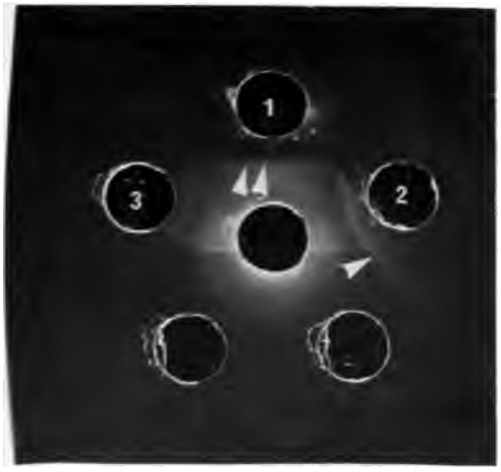


FIG. 4. Double immunodiffusion between rabbit antiserum to leucocyte elastase and SE. Center well, 8 μl of antiserum; well 1, 3.0 μg of elastase isozymes; well 2, 20 μl of acrylamide gel extract containing SE; well 3, 20 μl of Amicon filtrate (see Methods). The pattern was amplified by secondary diffusion against goat antirabbit immunoglobulin antiserum. For explanation of arrows, see text. (Note that the hexagonal-shaped zone of precipitation surrounding the center well is an artifact of the amplification procedure, which occurred despite extensive washing of the agarose prior to its treatment with the goat antiserum.)

TABLE I. DIFFERENTIAL COUNT OF BONE MARROW LEUCOCYTE PREPARATIONS.[a]

	Percentage of total cells			
Cell type	Marrow 1		Marrow 2	
Blasts		3.5		0.5
Promyelocytes		0.5		0.5
Myelocytes		8.5		12.0
Metamyelocytes		34.5		24.5
Total Immature	47.0		37.5	
Bands		21.0		13.0
Polymorphs		22.5		39.5
Total Mature	43.5		52.5	
Eosinophils		1.0		0.5
Lymphocytes		6.5		8.5
Erythrocytes		2.5		1.5

[a] Cells were smeared, stained, and counted at the final stage in their processing. This was after removal of most erythroid precursors and mature red cells by hypotonic lysis, and of platelets by differential centrifugation.

A second point raised by our results is that the presence of SE in functionally immature leucocytes, which do not yet have active elastase isozymes, suggests that SE may be a precursor or zymogen form of these enzymes. The observation of antigenic cross-reactivity between SE and the elastase isozymes of mature cells is consistent with the above interpretation. The fact that SE is Triton-activatable suggests that it may be an aggregated precursor form of the isozymes, and its slow migration into 13% acrylamide gels also supports this view. Several trials with elastin-containing acrylamide gels, prepared as described by Taylor and Crawford (9), failed to reveal lytic activity of SE against bovine ligament elastin; whereas in this test all three elastase isozymes were active. SE has the specificity required to attack elastin, since it hydrolyses Ac-L-Ala-L-Ala-L-Ala-1-ONap, a highly specific elastase synthetic substrate (unpublished observations of the present authors and (5)). However, an aggregated form of the enzymes might be sterically prevented from interacting with macromolecular substrates like elastin, while still being able to attack low MW substrates. On the other hand, the relative resistance of SE to low MW chloromethyl ketone inactivators is difficult to interpret. Many questions remain concerning the properties and function of SE in human neutrophil leucocytes, and further study will be required to resolve them.

Summary. Granules of human peripheral blood leucocytes contain four well-characterized elastase isozymes and one or two slow-moving elastaselike esterases (SE) which have not been as well characterized. SE are capable of hydrolyzing typical elastase synthetic substrates such as *N*-acetyl-DL-alanine-α-naphthyl ester (Ac-DL-Ala-1-ONap) and *N*-*t*-butyloxycarbonyl-L-alanine-*p*-nitrophenyl ester (Boc-Ala-ONp), but unlike the highly basic elastase isozymes, SE barely migrate into 13% acrylamide gels during cationic electrophoresis at pH 4.3. Hydrolysis of Ac-DL-Ala-1-ONap by SE requires the presence of Triton in the gel, and hydrolysis of Boc-Ala-ONp by the same enzyme(s) is also enhanced in the presence of the detergent. Triton is not required for these activities, in the case of the elastase isozymes. Diisopropylfluorophosphate (Dip-F) inactivates both SE and the elastase isozymes, whereas Ac-$(Ala)_2$-Pro-AlaCH_2Cl (a powerful inactivator of the leucocyte elastase isozymes at 10^{-4} *M* concentration) does not inactivate SE at the same concentration. Immunochemical studies revealed antigenic cross-reaction between the rapidly migrating leucocyte elastase isozymes and SE. Two preparations of leucocyte granules from nonleukemic bone marrow cells showed no activity of the rapidly migrating elastase isozymes, but did contain SE activity. SE may be a precursor or zymogen form of the elastase isozymes, present in immature cells and partly retained through later stages of development.

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Correlation of Plasma Lysosomal Enzyme Levels with Hepatic Reticuloendothelial Function after Trauma[1] (39323)

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Reticuloendothelial system (R.E.S.) phagocytic failure has been implicated as an important event in the pathogenesis of irreversibility following various forms of experimental shock. The role of the R.E.S. in shock survival is based primarily on four lines of evidence: (a) R.E.S. function deteriorates during shock (1-5); (b) survival following shock has been directly related to the degree of postshock R.E.S. recovery (2, 4); (c) survival is reduced when R.E.S. phagocytic activity is depressed prior to shock (1, 4, 5); (d) survival is increased when R.E.S. phagocytic activity is stimulated prior to shock (1, 4, 5). While the exact mechanism by which the R.E.S. exerts this protective defense role is not known, emphasis has been placed on the ability of the R.E.S. to remove and prevent the accumulation of particulate and toxic material in the circulation. Such potentially deleterious agents appear to include microthrombi, bacteria, endotoxin, damaged platelets, and lysosomal enzymes (5-10). Thus, it has been postulated that there is a failure in the mechanisms which clear toxic material from the blood during shock at a time when the elaboration of such material is greatly accelerated.

The present study was carried out to evaluate this concept by determining if a relationship exists between the plasma accumulation of one of these potentially toxic agents, i.e., lysosomal enzymes, and hepatic Kupffer cell phagocytic capacity following traumatic shock.

Methods. Male Sprague-Dawley rats (200-300 g) anesthetized with sodium pentobarbital (20 mg/kg) were subjected to Noble-Collip drum trauma (11) at 40 rpm. This trauma model allows the application of a highly standardized insult. On the basis of 24-hr survival it was determined that 300 revolutions (rev) resulted in less than 5% mortality (defined as sublethal), while 500 and 700 rev resulted in approx 50 and 95% mortality, respectively.

The test colloid employed to evaluate *in vivo* hepatic Kupffer cell clearance capacity was the gelatinized ^{131}I-"R.E.-test-lipid emulsion" as previously described (12). The emulsion was injected iv in both normal and post-trauma rats at a dose of 50 mg/100 g and hepatic Kupffer cell uptake evaluated 15 min following injection. The liver was rapidly excised and rinsed in chilled isotonic saline. The total organ was weighed and random aliquots removed for quantification of ^{131}I colloid activity as previously described (12). Results were expressed as milligrams of test colloid phagocytized per gram (mg/g) and per total organ (mg/TO).

Plasma levels of the lysosomal enzymes, cathepsin, and acid phosphatase, were determined using the methods described by Gianetto and DeDuve (13) and Smith and Filkins (14), respectively. For cathepsin determinations a 2% solution of denatured hemoglobin was used as the substrate and the reaction was carried out at pH 3.6. The aromatic degradation products were measured by the Lowry protein method using a tyrosine standard. The acid phosphatase assay was carried out at pH 5.0 with determination of the amount of inorganic phosphorus removed from the beta-glycerophosphate substrate.

Quantification of radioactivity was achieved with a Searle Analytic deep well autogamma scintillation counter. All samples were counted in duplicate with independent isotopic standards prepared for each experiment. Data were analyzed with a PDP/12 digital computer by the unpaired Student's t test placing the confidence level at 95%. Determination of correlation coef-

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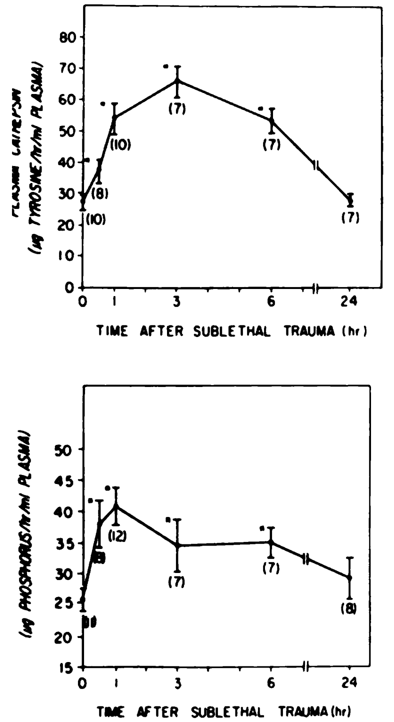
, and line placement by the method
t squares was also done with com-
assistance. Data are presented as
± standard error.

'ts. The temporal changes in plasma
in and acid phosphatase levels after
al trauma (300 rev) is presented in
Both enzymes followed similar pat-
f change with an initial increase at
and a return to control levels by 24
trauma. Plasma cathepsin reached a
m level equivalent to 144% of con-
3 hr while a maximum 59% elevation
phosphatase was observed at 1 hr
auma.



1. Temporal alterations in plasma cathepsin
phosphatase following sublethal trauma (300
ta are expressed as the mean ± standard error
number of animals indicated in parentheses.
(*) indicates significant ($P < 0.05$) difference
trauma control levels.

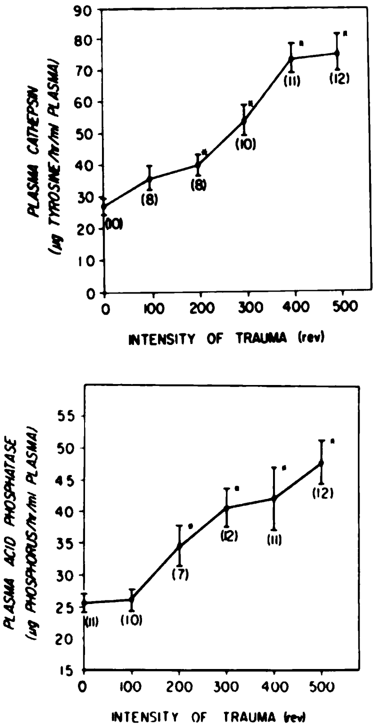


FIG. 2. Temporal alteration in plasma cathepsin and acid phosphatase at 60-min post-trauma with various intensities of trauma (100–500 rev). Data are expressed as mean ± standard error with the number of animals indicated in parentheses. Asterisk (*) indicates significant ($P < 0.05$) difference from pretrauma control levels.

na levels of cathepsin and acid phos-
: at 60-min post-trauma increased
sively with increments in trauma in-
(Fig. 2). Plasma cathepsin showed a
atively greater increase following
than acid phosphatase. Thus, 1 hr
00 rev (LD_{50}) cathepsin increased
while acid phosphatase increased 92%. The difference in the magnitude of change of these two enzymes is consistent with previous findings during hemorrhagic hypotension in the dog (9). In an attempt to correlate these enzyme alterations with the functional state of the hepatic Kupffer cell clearance mechanism, the functional state of the hepatic R.E.S. was evaluated over a similar time course.

Hepatic phagocytosis after sublethal trauma showed a maximum depression at 1 hr with recovery to pretrauma levels by 24 hr (Table I). With increasing trauma intensity there was a progressive decrease in hepatic phagocytosis 60-min post-trauma on both a per gram and total organ basis (Table I). Previous evaluation of other R.E. organs (spleen, bone marrow, and lung) during a period of Kupffer cell depression revealed increases of colloid localization in these extrahepatic sites as the half-time for colloid clearance from the circulation increased (12, 15–18).

TABLE I. Hepatic Kupffer Cell Phagocytic Activity following Traumatic Shock as a Function of Post-Traumatic Interval and Degree of Injury.[a,b]

	Number of animals	Kupffer cell phagocytosis[c]			
		mg/g	%C	mg/TO	%C
Post-Trauma interval					
Pretrauma controls	7	3.35 ± 0.19	100	46.39 ± 3.37	100
0.5 hr	7	1.89 ± 0.21[d]	56	27.17 ± 3.43[d]	59
1 hr	6	1.49 ± 0.07[d]	45	20.26 ± 0.83[d]	44
3 hr	7	1.77 ± 0.12[d]	53	24.54 ± 2.33[d]	53
6 hr	4	2.19 ± 0.30[d]	65	28.01 ± 3.71[d]	60
24 hr	8	3.40 ± 0.22	102	43.87 ± 2.40	95
Trauma intensity					
Pretrauma controls	5	4.69 ± 0.18	100	57.72 ± 7.64	100
100 rev	5	3.67 ± 0.50	78	41.68 ± 6.14	72
200 rev	5	4.30 ± 0.46	92	53.64 ± 7.13	93
300 rev	5	2.90 ± 0.53[d]	62	30.35 ± 4.96[d]	53
400 rev	5	2.97 ± 0.31[d]	63	40.89 ± 4.56[d]	71
500 rev	5	2.32 ± 0.17[d]	50	27.82 ± 4.01[d]	48

[a] In the time course study (0–24 hr), rats were subjected to 300 rev of NCD trauma and evaluated with a 50 mg/100 g test dose of the colloid at times indicated.

[b] In the trauma intensity series (0–500 rev), rats were evaluated at 60-min post-trauma with the test colloid.

[c] Data is presented as means ± SE of the mean. Values are presented as milligrams test emulsion phagocytized per gram of liver (mg/g) and per total organ (mg/TO) on a wet weight basis. %C is the percentage of pretrauma control values.

[d] Significantly different from control group ($P < 0.05$).

Numerous studies have indicated that increased plasma lysosomal enzyme activity and decreased phagocytic capacity of the R.E.S. are related to shock severity (1–5, 8–10); however, a direct relationship between these two parameters has not been previously demonstrated. Figure 3 documents this relationship and reveals a significant correlation between post-trauma hepatic phagocytosis and the plasma levels of both cathepsin ($P < 0.05, r = -0.604$) and acid phosphatase ($P < 0.05, r = -0.634$). This inverse relationship between hepatic phagocytosis and plasma lysosomal enzyme levels was observed with the time course changes after sublethal trauma as well as with the changes 1 hour after various intensities of trauma.

Discussion. The observed changes in hepatic R.E. function following trauma are consistent with previous data which demonstrated an initial fall in R.E.S. function followed by a recovery or even hyperactivity within 24 hr (2–4, 16, 17). Similarly, the changes in plasma lysosomal enzyme levels observed were qualitatively similar to previous findings (8–10). The present study extends previous investigations in terms of quantifying the time course of changes following traumatic shock while further documenting the relationship between the degree of trauma and both the stability of the R.E.S. as well as the plasma lysosomal enzyme levels.

There are at least four possible interpretations of the present observation of the correlation of plasma lysosomal enzyme levels with R.E.S. function after trauma. These include: (a) Kupffer cell function decreases and plasma lysosomal enzyme levels increase independently as a function of the degree of tissue injury with no cause and effect relationship; (b) lysosomal enzymes are cleared from the blood by the R.E.S., and Kupffer cell clearance depression allows enzyme accumulation in the blood following trauma; (c) high plasma lysosomal enzyme levels in the post-trauma state depress R.E.S. phagocytic function; (d) enzymes released into the plasma following trauma are released primarily from injured R.E. cells and thus reflect the stability of this cellular system.

The possible clearance of lysosomal enzymes by the R.E.S. is supported by the finding that exclusion of the liver from the circulation via a porta-caval shunt preparation results in a greatly prolonged retention

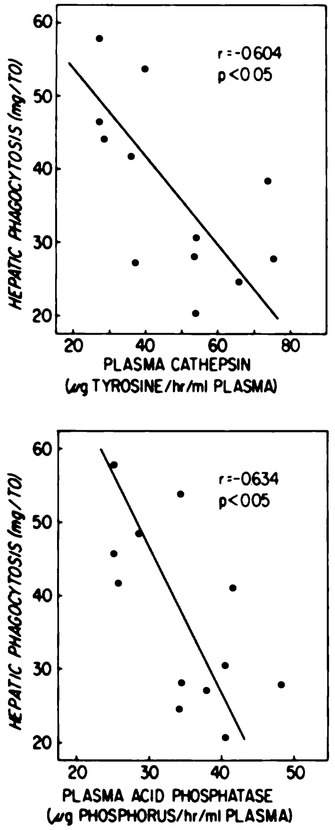


FIG. 3. Correlation between hepatic Kupffer cell phagocytosis and plasma cathepsin (upper) and plasma acid phosphatase (lower) after trauma. Plotted values are means of data obtained at various times ($^1/_2$-24 hr) after sublethal trauma (300 rev) and 1 hr after various levels of trauma (100-500 rev).

of lysosomal enzymes in the blood (19, 20). Depression of R.E.S. function may therefore result in a greater accumulation of these hydrolase enzymes in the plasma as a result of decreased clearance. Trauma may induce alterations in the splanchnic microcirculation (21, 22) which could result in: (a) R.E.S. failure by limiting delivery of particulate material to phagocytic cells; (b) ischemic injury of hepatic Kupffer cells; and (c) release of lysosomal enzymes from ischemic splanchnic tissue. Additionally, high circulating lysosomal enzyme levels have been shown to decrease splanchnic blood flow (19).

R.E. function and lysosomal enzyme levels may change independently of each other or in a related manner as a result of tissue injury. Lysosomal hydrolases may be released as a direct consequence of cellular injury while R.E. depression may result from opsonin depletion (17, 18). Alternatively, since lysosomal enzymes are released from macrophages during phagocytosis (23), and since R.E. depression is observed following phagocytosis (12) these phenomenon may occur secondarily to an accumulation of phagocytizable debris subsequent to trauma. While R.E. cells do contain a high concentration of lysosomal enzymes (24), the magnitude of the enzyme release coupled with the size of the R.E. cell population cast doubt that all the enzyme activity is released from ischemic or injured R.E. cells. Indeed, the flux of such enzymes from the intestine and pancreas, organs with minimal R.E. activity, would further negate this possibility (25). Lysosomal hydrolases may be released from injured R.E. cells, but histological evidence indicates that Kupffer cell damage is only present with irreversible shock (1).

Thus, the present study has shown that there is a correlation between hepatic Kupffer cell function and the accumulation of lysosomal enzymes in the circulation following traumatic injury. Since previous studies have demonstrated that R.E.S. function correlates well with shock survival (2, 4), the observed relationship between circulating lysosomal enzyme levels and R.E.S. function warrants further investigation.

Summary. Plasma lysosomal enzyme levels and hepatic phagocytosis were determined following Noble-Collip drum trauma in the rat. Circulating cathepsin and acid phosphatase activity increased after sublethal trauma (300 rev), reaching maximal levels at 1-3 hr and returning to pretrauma levels at 24 hr after trauma. Hepatic phagocytosis was decreased maximally at 1 hr and recovered to control levels at 24 hr after sublethal trauma. Increasing trauma intensity (100-500 rev) resulted in a progressive failure in hepatic Kupffer cell phagocytosis and a progressive increase in plasma lysosomal enzyme levels when tested at 60-min post-trauma. A significant inverse correlation was found between the plasma lysosomal enzyme levels and Kupffer cell phagocytosis after trauma. The functional significance of the relationship between these two

parameters and its importance in shock survival remain to be determined.

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OS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 47-51 (1976)

Effect of Fasting and Insulin on the Glucagon-Induced Orthophosphate Incorporation to the Isolated Perfused Rat Liver[1] (39324)

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revious experiments (1) it was shown lucagon, epinephrine, cAMP, and dB- ? were all able to increase the incorpo- of orthophosphate to the isolated per- fed rat liver. This phenomenon may in, in part, the well-known hypophos- mic effect exhibited by these glycogen- factors (2–6). The functional meaning increased orthophosphate incorpora- the liver has not been clarified yet. It en postulated that such incorporation be related to an increment of phos- requirements during glycogenolysis n the other hand, in the last few years il investigators have pointed out that on and cAMP may activate protein horylations in the liver (7–12), and ffect could also have some relationship he above mentioned phenomenon.

r previous experiments (1) were per- d in fed rat livers in which a clear se in spontaneous or glucagon-in- glycogenolysis was evidenced; there- it was considered of interest to study fect of glucagon in two experimental tions which inhibit glycogenolysis gh different mechanisms: fasting and administration.

terial and methods. Male Sprague- y rats (300–350 g body weight) bred r Institute were used in the experi- . Livers were perfused by means of a usly described technique (1, 13) using of perfusate which consisted of Krebs- r-bicarbonate containing 3 g/100 ml ine serum albumin (Armour Fraction) mg/100 ml of glucose, 1.7 mg/100 ml ffered sodium orthophosphate, and vol % of washed rat erythrocytes. rin (500 U) was added at the beginning of the experiments. The perfusate was equilibrated with a 95% O_2–5% CO_2 gas mixture, which was also used in the oxygenator. pH was adjusted to 7.40 and the temperature was kept at 37°. Gross and ultramicroscopic observation as well as adequate bile flow were indicative of good liver preservation.

After 15-min perfusion, 70 μCi of high-activity [^{32}P]orthophosphoric acid (Amersham/Searle, PBS-1200 mCi/mmol) was added to the perfusate (0 time). Thereafter samples were withdrawn every 15 min, up to 120 min, and collected in ice-chilled test tubes. The radioactivity of the samples was determined in duplicate 0.1-ml perfusate aliquots plated in plastic cups and counted up to 10,000 in a Nuclear Chicago gas flow counter. The number of counts registered in the 45-min samples was taken as 100% for the expression of the total radioactivity of the subsequent samples. The percentage of ^{32}P incorporated into the liver was estimated by substraction and expressed per 10 g of fresh tissue.

Perfusate glucose concentrations were determined in duplicate by a commercial glucoxidase procedure (Glucostat, Worthington). The figures were corrected for changes found in blank experiments performed in absence of the liver. Results were expressed in term of differences from 45-min values, mg/100 ml/10 g of fresh liver.

Perfusate plasma Pi was determined in duplicate with the method of Martin and Doty (14). Figures were corrected for blank values and expressed in terms of differences with respect to the 45-min values (mg/100 ml). In the absence of the liver there is some decrease of perfusate plasma Pi due to incorporation to red cells; the differences against the 45-min values were: at 60 min, −0.12 ± 0.14; at 75 min, −0.28 ± 0.09; at 90 min, −0.41 ± 0.15; and at 120 min, −0.41 ± 0.17 (N = 12).

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The radioactivity of the final colored solutions obtained after the Pi determinations were measured in order to estimate the specific activities of the samples. For this purpose, 0.5-ml aliquots were plated in plastic cups and counted to 10,000 counts in a Nuclear Chicago gas flow counter. The specific activities were expressed in counts per milligram of Pi. The specific activity obtained at 45 min was taken as 100%, and the changes of the following samples were calculated as the percentage of this figure.

The pH was determined at 0, 60, and 120 min; some degree of alkalinization was observed at 0 min, but thereafter it was maintained close to 7.40.

Fasted animals were deprived of food during the 24 hr preceding the experiments. Fed animals had free access to food up to the time of the experiments. Water was *ad libitum* in both experimental groups. Glucagon, when administered, was used at the dose of 2 μg and was infused continuously to the perfusate from 45 to 90 min.

Experiments on the effect of insulin were carried out on fed rat livers and the hormone (250 or 500 mU) was continuously infused to the perfusate from 0 to 90 min. Glucagon was used as previously described for the fasting experiments. Insulin-free glucagon, generously provided by Ely Lilly Co., was dissolved in a 0.2 *M* glycine-NaOH buffer, pH 9, containing 1 g/100 ml of bovine serum albumin; working solutions were prepared in saline containing 1 g/100 ml of bovine serum albumin. Glucagon-free insulin, also furnished as a gift by Ely Lilly Co. as a 40 U/ml solution, was dissolved in saline containing 0.1 g of bovine serum albumin, pH 3.5. The selected doses of both hormones were made up in 1-ml vol, and the same amount of saline was injected in the control experiments. The solutions were introduced in the overflow tube of the perfusion apparatus in order to allow for mixture with the bulk of the perfusate before reaching the liver.

The Student's *t* test was used to appraise the statistical significance of the results, and $P = 0.05$ was taken as the limit of significance.

Results. The effect of fasting on the studied parameters is shown in Fig. 1. It is interesting, in the first place, to compare control fasted rat livers with control fed rat livers; it can be seen that the spontaneous glucose and orthophosphate liberation which took place from fed rat livers did not occur in the fasted rat livers. With respect to the effect of glucagon, it can be observed that in the fasted rat livers no significative liberation of

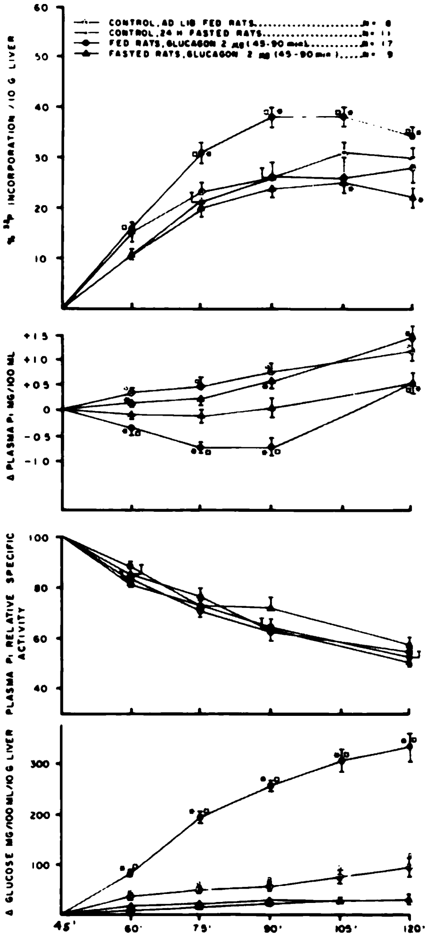

FIG. 1. Effect of glucagon (2 μg) on [^{32}P]orthophosphate incorporation into the isolated perfused rat liver, perfusate plasma inorganic orthophosphate, relative specific activity of the same, and glucose release from the organ. Glucagon was continuously infused into the perfusate for 45 min beginning at 45 min. Comparison between livers from 24-hr fasted rats and *ad libitum* fed rats.

Statistically significant differences: *, Glucagon-fed rat livers vs control fed rat livers; ○, control fed rat livers vs control fasted rat livers; □, glucagon-fed rat livers vs glucagon-fasted rat livers.

glucose or orthophosphate incorporation takes place under the action of the hormone.

Figures 2 and 3 show the effect of insulin and of glucagon plus insulin. Figure 2 pertains to the 250-mU insulin dose: as is well known, insulin, if given since the beginning of the perfusion reduces the glucose output (15, 16); it also inhibits the spontaneous orthophosphate efflux (16, 17). The effect of glucagon on glucose liberation is also reduced by insulin (16, 18); however, it is possible to observe that glucagon-induced orthophosphate incorporation to the liver is not inhibited by insulin. No significant difference was registered among the groups in relation to Pi specific activities. Figure 3

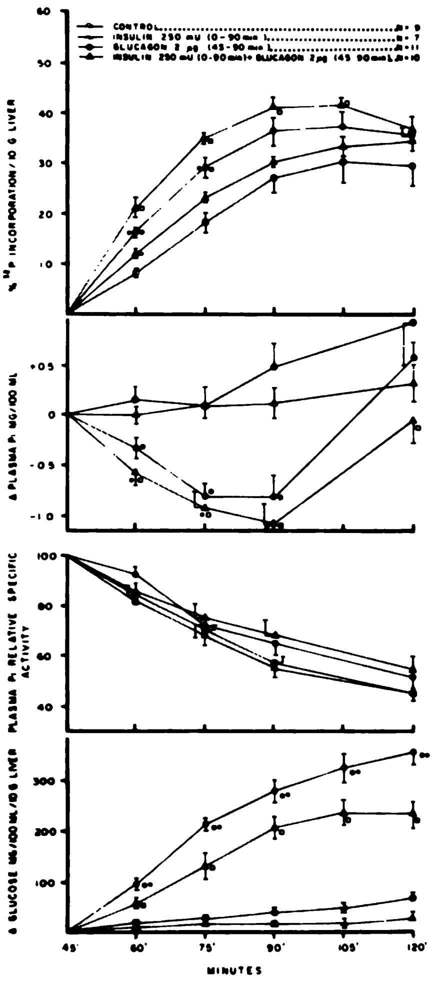

FIG. 2. Effect of insulin (250 mU), continuously infused from 0 to 90 min, glucagon (2 μg) continuously infused from 45 to 90 min, and both types of treatments administered simultaneously to perfusate, on [^{32}P]orthophosphate incorporation into the isolated perfused rat liver, perfusate plasma inorganic orthophosphate, relative specific activity of the same, and glucose release from the organ.

Statistically significant differences: *, Glucagon vs control; O, insulin vs control; □, glucagon + insulin vs control; ★, glucagon vs glucagon + insulin.

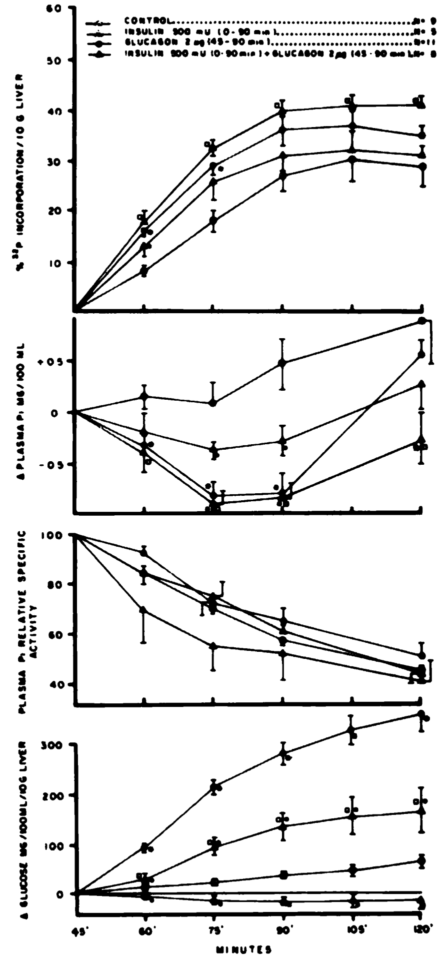

FIG. 3. Effect of insulin (500 mU) continuously infused from 0 to 90 min, glucagon (2 μg) continuously infused from 45 to 90 min, and both types of treatments administered simultaneously to perfusate, on [^{32}P]orthophosphate incorporation into the isolated perfused rat liver, perfusate plasma inorganic orthophosphate, relative specific activity of the same, and glucose release from the organ.

Statistically significant differences: *, Glucagon vs control; O, insulin vs control. □, glucagon + insulin vs control; ★ glucagon vs glucagon + insulin.

refers to the 500-mU dose of insulin. The results are similar to the ones described with the 250-mU dose, but the changes observed are more evident.

Discussion. It is well known that the reduction of the liver glycogen stores, determined by fasting in the absence of gluconeogenic substrates, inhibits the spontaneous or glucagon-induced glucose release from the perfused rat liver. The spontaneous glucose liberation that takes place from the fed perfused liver has been attributed to anoxia, surgical manipulations (19), and sympathetic discharges originated by surgery (20). However, it could also be possible that insulin lack may play some role, since the phenomenon may be inhibited by the administration of this hormone (15, 16) and is increased by the administration of anti-insulin serum (16). The mechanism of the spontaneous orthophosphate release is not known, but the work of Jefferson *et al.* (16) and our own experiments (17) have shown that it can be inhibited by insulin administration. A similar situation has been mentioned in connection with the perfused rat heart (21, 22). It is interesting that fasted rat perfused livers do not show a significant spontaneous orthophosphate release into the perfusate. The facts that in the fed rat perfused liver there is a concomitant output of glucose and orthophosphate and that both phenomena may be inhibited by insulin administration suggest the possibility that both effects may be functionally related. In the fed rat perfused liver, however, glucagon induces glycogenolysis and more glucose is released, while, at the same time, orthophosphate is incorporated into the organ. In the fasted rat perfused liver, both phenomena are inhibited. Hence, regarding orthophosphate exchange, in the fed perfused rat liver, spontaneous glycogenolysis is accompanied by orthophosphate release, and glucagon-induced glycogenolysis is accompanied by orthophosphate incorporation. This discrepancy could be explained by differences in the action of *in situ* liberated catecholamines and glucagon; however, it should be mentioned in this regard, that in previous experiments (1), we have shown that at least one of the catecholamines, epinephrine, is also able to induce orthophosphate incorporation into the perfused fed rat liver. It could be possible that the main factor in spontaneous glucose and orthophosphate liberation from fed rat liver is the lack of insulin, since this hormone acts on the orthophosphate liver exchange producing retention of the anion (1); however, this mechanism is not easily understandable in terms of cAMP levels regulated by the glucagon–insulin interplay.

In the experiments on insulin-inhibition of the glucagon-induced glucose release, the incorporation of orthophosphate to the liver was not affected, a result which dissociates both phenomena and also suggests a different mechanism of regulation. The inhibition of glucose liberation falls in the above mentioned interplay, but the same cannot be said for orthophosphate incorporation. A similar situation has been mentioned in connection with other glucagon actions (23, 24). Effects of insulin, independent of the cAMP levels, have been also detected (23, 25).

More experimental work is required to further our understanding of the regulation of liver orthophosphate exchange in connection with the action of glucagon and insulin. Studies in course in our laboratory concerning the role of modifiers of the adenylate system and adrenergic blockers, may provide additional information on this matter.

Summary. Isolated perfused fed rat livers spontaneously liberated glucose and orthophosphate to the medium; 24-hr fasted rat livers did not exhibit these phenomena. In perfused fed rat livers, glucagon (2 μg) increased glucose output and promoted orthophosphate incorporation. In perfused fed rat livers, insulin (250 or 500 mU) inhibited the spontaneous liberation of glucose and orthophosphate. Comparable doses of insulin significantly reduced the glucagon (2 μg)-induced increase in glucose output from perfused fed rat liver, but did not affect orthophosphate uptake by the organ.

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O_2 Extraction of Right and Left Ventricles (39325)

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Simultaneous measurements of O_2 extraction, and hence $\dot{V}O_2$ are not available in the literature for the right ventricle working *in situ*. This information is essential for computations of tissue pO_2 gradients in right ventricle, and for comparison of the function of the two ventricles.

Materials and methods. Mongrel dogs were anesthetized with Na pentobarbital, 60 mg/kg body weight. They were ventilated at a rate which maintained $PaCO_2$ and pHa within normal limits. Because of large $\dot{V}A/Q$ inequalities during cardiac manipulation in open-chest dogs, it was necessary to use 100% O_2 as the inspired gas to insure full saturation of hemoglobin. From 250-500 ml of dextran were given to compensate for blood loss and the hemodynamic effects of thoracotomy. The right ventricular free wall was exposed, and a catheter was inserted into the coronary sinus via the right jugular vein. The catheter tip was positioned to sample blood from the posterior aspect of the left ventricle. The free wall of the right ventricle is drained by several small anterior cardiac veins (1). One of these was punctured with a #24 needle attached to a flexible polyvinyl catheter. Care was taken to insure that the vein selected was well removed from the interventricular septum. Blood was drawn simultaneously from the two venous catheters into oiled glass syringes which contained dry heparin. Care was taken to discard blood which had been in contact with air or saline in the dead space of the catheters. An arterial sample was also drawn simultaneously, or immediately after venous samples were collected. Hemoglobin percentage saturation and blood gases were measured with an Instrumentation Laboratories system; the co-oximeter was calibrated for dog blood. Arterial pressure was monitored on an oscilloscope. It remained stable and within normal limits throughout.

Results and discussion. Table I summarizes results of 11 measurements in four dogs. Replicates for each dog were averaged, and these average values used to calculate means and standard errors. Extraction was about 2.5 vol% higher in blood from left ventricle. Note that the difference in extractions is about the same over a 2.5-fold range of arterial O_2 content. On the other hand, pO_2 was about 6 mm Hg higher in blood draining right ventricle.

In man (2) and dog (1, 3) blood flow/100 g in right ventricle is about 60% that of the left. Combining this figure with the ratio of O_2 extraction in right and left ventricles from Table I:

$$\dot{V}O_2/100\text{ g RV} \simeq 0.6\,Q/100\text{ g LV} \times [0.75\,(CaO_2 - CvO_{2LV})].$$

Thus, in large animals $\dot{V}O_2/100$ g of right ventricle is 40–50% of the $\dot{V}O_2/100$ g of left ventricle. Since end-capillary pO_2 is higher in right ventricle, and right ventricular $\dot{V}O_2$ is lower, one would expect intracellular pO_2 to be higher in right ventricle. However, according to Whalen and co-workers (4), intracellular pO_2 is the same in the two ventricles. This can only be true if diffusion distances are larger in right ventricle than left. This appears to be the case (5).

To our knowledge the only studies of O_2 extraction of right ventricle previous to ours have been made during cardiac by-pass (1, 6). They therefore do not pertain to the working right ventricle. Moreover, measurements on by-pass are subject to criticism on technical grounds for right ventricular venous drainage was collected from the right ventricular cavity (1, 6). Since arterial as well as venous connections with the lu-

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TABLE I. O_2 EXTRACTION BY RIGHT AND LEFT VENTRICLES.

		Arterial blood				Anterior cardiac vein			Coronary sinus			Average differences	
Dog number	Sample number	Hb (g%)	PaO_2 (mm Hg)	CaO_2 (Vol%)	pH_a	$P_{RV}O_2$ (mm Hg)	$C_{RV}O_2$ (Vol %)	Extraction (Vol%)	$P_{LV}O_2$ (mm Hg)	$C_{LV}O_2$ (Vol %)	Extraction (Vol%)	RV-LV PO_2 (mm Hg)	LV-RV extraction (Vol%)
1	1	7.2		10.63	7.47	31.3	–	–	22.7	3.16	7.47		
	2					34.5	5.97	4.66	23.7	3.25	7.38	9.30	2.62
	3		488			31.2	5.78	4.85	22.7	3.25	7.38		
2	1	12.3		16.84	7.44	26.3	7.83	9.01	19.6	5.17	11.67		
	2		450			26.1	–	–	20.6	5.17	11.67	5.17	2.32
	3					24.1	6.99	9.85	20.8	5.0	11.84		
3	1	14.3	130	18.98	7.31	37.0	11.42	7.56	30.2	8.52	10.46		
	2	14.2	123	18.96	7.32	37.5	11.22	7.74	30.0	8.52	10.44	6.87	2.96
	3	14.4	127	18.81	7.33	36.5	11.61	7.20	30.2	8.33	10.48		
4	1	19.9	313	27.34	7.38	41.5	19.51	7.83	34.3	15.18	12.16		
	2	20.2	292	27.01	7.37	33.4	14.91	12.10	33.3	14.91	12.10	3.60	2.16
Mean		13.5	340	18.40	7.40	33.07	10.48	7.92	26.82	7.31	10.52	6.25	2.52
± SE												1.22	0.18
												$P < 0.05$	$P < 0.001$

men exist (1), the O_2 content of blood from the lumen may be erroneously high. Measurements during by-pass are widely used to estimate the O_2 cost of resting metabolism, excitation, and internal work. We therefore urge that right ventricular O_2 extraction during by-pass be reinvestigated by the venipuncture method used in the present study.

Summary. O_2 extraction was measured simultaneously in right and left ventricles of dogs. Extraction was about 2.5 vol% higher in left ventricle. This figure, together with flow measurements of others (1-3), indicates that $\dot{V}O_2/100$ g is at least twice as great in left ventricle as in right.

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Progesterone Synthesis by Luteal Mitochondria *in Vitro*[1] (39326)

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It is generally accepted that luteal mitochondria contain the enzyme complex required for the conversion of cholesterol to pregnenolone (1). However, it has not been possible to determine if these mitochondria also contain the enzymes required for progesterone synthesis, or if the synthesis reported by other investigators (2-4) was due to contamination with microsomal enzymes. We have investigated this problem using highly purified luteal mitochondria prepared by isopycnic zonal centrifugation, and we have demonstrated that these organelles contain the enzymes required for progesterone synthesis.

Materials and methods. Mitochondria were prepared from bovine corpora lutea within 1 hr after slaughter. The tissue was passed through a cold tissue press (Harvard Apparatus Company) and then homogenized in 0.25 *M* sucrose, pH 7.4, using a chilled homogenizer (No. 22 Duall, Kontes Glass Co.) with the pestle rotating at 1000 rpm. Heavy debris and nuclei were removed by centrifuging the homogenate two times at 1000 *g* for 10 min. The supernatant was then respun at 6000 *g* for 10 min and the surface of the pellet was rinsed lightly with 0.25 *M* sucrose. The pellet was resuspended and centrifuged two more times and resuspended in 10 ml of 0.25 *M* sucrose. The mitochondria were purified further by isopycnic zonal centrifugation, as follows: the mitochondrial suspension was introduced into a Beckman Ti-14 zonal rotor containing a discontinuous-continuous sucrose gradient, as previously described (5). The rotor was spun at 25,000 rpm for approx 45 min to reach an $\omega^2 t$ value of 1.9×10^{10}, as determined by a Beckman $\omega^2 t$ integrator. At the end of the run, the rotor was unloaded and 26 fractions of 25 ml each were collected. The fractions were diluted with 15 ml of doubly distilled water and centrifuged at 7500 *g* for 15 min. The pellets were resuspended in 0.25 *M* sucrose for biochemical studies.

Cytochrome oxidase activity (a marker for the inner mitochondrial membrane), protein, and nucleic acid content were determined as previously reported (6). Mitochondrial steroidogenic activity was determined by a modification of the method of Robinson and Stevenson (7) which measures the percentage of [4-^{14}C]cholesterol converted to [4-^{14}C]pregnenolone and [4-^{14}C]progesterone. In our studies, 20,000 cpm of [4-^{14}C]cholesterol and 2 mg of mitochondrial protein were used per assay, and succinate was used as a substrate.

Results. Isopycnic zonal centrifugation resulted in a concentration of the mitochondria in the part of the gradient around 44% (w/w) sucrose, as indicated by the distribution of cytochrome oxidase activity (Fig. 1).

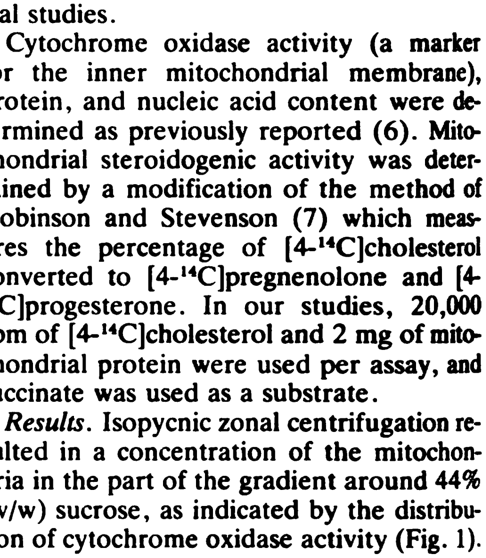

FIG. 1. Distributions of cytochrome oxidase activity and protein after isopycnic zonal centrifugation. Mitochondrial samples were prepared from four to six isolated bovine corpora lutea by differential centrifugation and purified by centrifuging them in a Beckman Ti-14 zonal rotor containing a sucrose gradient ($\omega^2 t = 1.9 \times 10^{10}$). Twenty-six fractions (25 ml each) were collected, diluted with water, centrifuged at 7500 *g* for 15 min, and the pellets resuspended in 0.25 *M* sucrose. The experiments were done on three different mitochondrial samples prepared from pooled corpora lutea, and a representative set of data are presented here.

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[1] Aided by Grant No. AM 05474, National Institute of Arthritis, Metabolism and Digestive Diseases, and by the Women's Guild of Sinai Hospital.

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ı distribution was similar to that of rome oxidase activity, except for 'hat elevated values in the fractions ed at the lighter end of the gradient. ic cytochrome oxidase activity and c [4-^{14}C]pregnenolone and [4- ogesterone synthetic activities (i.e., y per milligram of protein) of com- fractions 16, 17, and 18 were higher hose obtained with mitochondria sep- by differential centrifugation alone : I). However, the DNA and RNA ıts per milligram of protein decreased tically after further purification of lu- ıitochondria by zonal centrifugation. yntheses of [4-^{14}C]pregnenolone and]progesterone were linear for at least n, with the [4-^{14}C]progesterone syn- rate being the faster of the two (Fig.

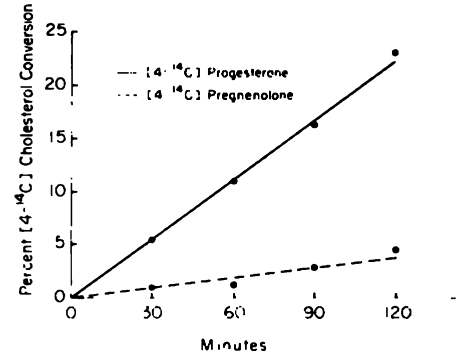

FIG. 2. The percentage of [4-^{14}C]cholesterol converted to [4-^{14}C]pregnenolone and [4-^{14}C]progesterone per time by luteal mitochondria purified by zonal centrifugation. The experiment was done on three different preparations of luteal mitochondria and a representative set of data is presented here.

ussion. The absence of nuclear and ıomal contamination of luteal mito- ria obtained by isopycnic zonal cen- ıtion is suggested by their low specific c acid contents, which are comparable ochondrial DNA and RNA values re- l by other investigators (8). Furthermore, the protein distribution obtained after zonal centrifugation suggests that the higher specific cytochrome oxidase activity and [4-^{14}C]pregnenolone and [4-^{14}C]progesterone synthesis rates seen in luteal mitochondria purified by zonal centrifugation were due to the loss of nonmitochondrial protein. The fact that the mitochondria had synthesized more [4-^{14}C]progesterone than [4-^{14}C]pregnenolone supports the generally held view that cholesterol conversion to pregnenolone is the rate limiting step of ovarian steroidogenesis (1, 2).

ABLE I. SPECIFIC CYTOCHROME OXIDASE :TIVITY, NUCLEIC ACID CONTENT, AND [4- 'C]CHOLESTEROL CONVERSION BY LUTEAL MITOCHONDRIA.

	Differential centrifugation[a]	Differential centrifugation followed by zonal centrifugation
rome oxidase ac- ity (U/mg)tein)[b]	17.5	23.4
µg/mg protein)	5.1	1.9
µg/mg protein)	16.2	6.5
age [4- ']cholesterol ıversion to:[c]		
]Pregnenolone	0.9	1.3
]Progesterone	6.8	9.5

tochondria isolated by differential centrifuga- ıne represent an aliquot of the sample prior to entrifugation. The experiments were done on lifferent mitochondrial preparations prepared ur to six bovine corpora lutea, and a representa- of data are presented here.

is defined as k (sec) × 10^{-4}.

rcentage [4-^{14}C]cholesterol conversion values ɔtained by incubating 2 mg mitochondrial sam- th 20,000 cpm [4-^{14}C]cholesterol for 60 min in sence of succinate.

Although the enzymes required for progesterone synthesis have been found in other subcellular fractions of luteal homogenates (9), it is likely that at least some of this activity is due to contamination from mitochondrial fragments. However, it is possible that two sites for progesterone synthesis may exist in luteal tissue. In contrast, thecal cells which primarily secrete estrogen have been reported to favor the conversion of pregnenolone to 17-OH progesterone via the Δ^5-pathway (10). Thus, mitochondria of these cells may not be able to synthesize progesterone.

In conclusion, our studies strongly suggest that the enzymes required for the conversion of pregnenolone to progesterone are present in highly purified mitochondria from bovine corpora lutea.

Summary. Mitochondria were prepared from bovine corpora lutea by differential centrifugation and were purified by isopycnic zonal centrifugation. A marked increase in specific cytochrome oxidase activity and a marked decrease in specific DNA and RNA content indicate that the procedure resulted in a highly purified preparation of mitochondria. These organelles had a higher rate of conversion of [4-^{14}C]cholesterol to [4-^{14}C]progesterone than did mitochondria separated only by differential centrifugation, suggesting that luteal mitochondria contain the enzyme systems required for progesterone synthesis.

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THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 57-60 (1976)

# of Immunostimulants on Resistance of Newborn Mice to Herpes Simplex Type 2 Infection (39327)

T E. STARR, AAROLYN M. VISINTINE, M. OUSAMA TOMEH, AND ANDRÉ J. NAHMIAS

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simplex virus (HSV) infection of :onates is associated with a high rate, and survivors frequently re neurological or ocular sequelae \lthough antiviral chemotherapy)deoxyuridine, cytosine arabino- denine arabinoside has been used 'eatment of neonatal herpes, the f these drugs is currently undeter- , 2). Other agents, which might 'us replication or bolster the new- mune system specifically or non- y, require investigation.

to the human newborn, who is 'eptible to viral dissemination than ividuals, the newborn mouse has ed to die when inoculated with virus which would not kill older i). Survival of newborn mice can :antly increased by the transfer of ıse peritoneal macrophages prior allenge (4). Since transfer of mac- is not readily feasible in human it was of interest to determine if ıich might stimulate macrophages cells involved in immunity would creased survival of newborn mice /ith HSV. Blaese has reported en- .rvival of newborn rats challenged *'ria monocytogenes* after adminis- a number of such agents (6). A HSV-2 was used in these studies ut three-quarters of HSV strains from neonates have been typed in atory as HSV-2, which is also the t often associated with genital in- ı adults (1, 2).

ls and methods. Mice. Outbred newborn Swiss mice were used. tter, pregnant mice were obtained Animal Farms, Prattville, Ala.) ates of delivery of the litters were

\ strain of HSV-2 isolated from a newborn with disseminated herpetic infection was used. A virus pool prepared from infected primary rabbit kidney (RK) cells had a titer of 4 × 10^6 plaque-forming units (PFU) per ml.

Immunostimulants. The following putative immunostimulants were employed: BCG, Tice strain, 2–8 × 10^8 viable organisms per ml (obtained from Dr. Roy Crispen, University of Illinois Medical Center, Chicago, Illinois); Levamisole, 2.5–25 mg/kg (Janssen R & D, Inc., New Brunswick, N.J.); Staphage lysate, undiluted (Delmont Laboratories, Swarthmore, Pa.); Typhoid vaccine 1–8 units/ml (Wyeth Laboratories, Marietta, Pa.); Brucella vaccine, 10^9 organisms per ml (obtained from Dr. Michael Blaese, NIH, Bethesda, Md.). BCG and levamisole were reconstituted or diluted with normal saline.

Antisera to HSV-2. Antiserum to HSV-2 was prepared in rabbits, as previously described (7), and had a neutralizing titer of 1:1024.

Inoculation of mice. One- to two-day-old litters of newborn mice were inoculated via the intraperitoneal (ip) route with 0.05 ml of one of the immunostimulants to be evaluated. In some experiments BCG was administered intradermally (id). Two, four, and six days later the animals were challenged ip with 10 LD_{50} of HSV-2 in 0.05 ml. Controls consisted of age-matched animals receiving only an immunostimulant, and age-matched animals receiving saline and then challenged with virus. In other experiments, the effect of HSV antiserum administered 24 hr following viral challenge was determined. Mice were observed daily for 3 weeks and the times of death recorded. *P* values were calculated by use of the chi-square test or the exact test.

Results. Determination of LD_{50}. Newborn and 6-week-old mice were inoculated with

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ɛd.

serial dilutions of the virus. The results (Fig. 1) are similar to previous findings obtained by others (4, 5) with HSV-1, in that newborn mice succumb when inoculated ip with doses of HSV-2 that do not kill adult mice. The LD_{50}s for newborn and adult mice, calculated by the Reed-Muench method, were 67 PFU and greater than 2×10^4 PFU, respectively.

Survival of newborn mice receiving immunostimulants. In preliminary experiments no protective effect could be demonstrated when undiluted BCG was administered either 2 or 4 days prior to viral challenge with 10 LD_{50} of HSV-2; 3 of 31 mice given BCG 2 days prior to challenge survived; 2 of 24 mice given BCG 4 days prior to challenge survived. When undiluted BCG was given 6 days prior to viral challenge, highly significant protection was observed ($P < 0.0005$) as shown in Fig. 2. No protective effect could be demonstrated when any of the other putative immunostimulants were administered 2, 4, or 6 days prior to viral challenge at the doses indicated. Deaths in control animals receiving any of the immunostimulants alone were not observed.

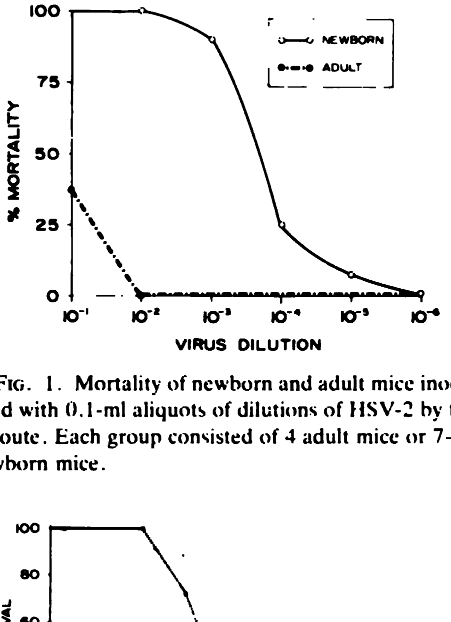

FIG. 1. Mortality of newborn and adult mice inoculated with 0.1-ml aliquots of dilutions of HSV-2 by the ip route. Each group consisted of 4 adult mice or 7-11 newborn mice.

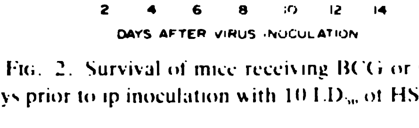

FIG. 2. Survival of mice receiving BCG or saline 6 days prior to ip inoculation with 10 LD_{50} of HSV-2

Effect of dose and route of administration of BCG. Litters of newborn mice were inoculated with 0.05 ml BCG, undiluted or diluted 1:5, by the ip or id route 6 days prior to viral challenge. Mice receiving undiluted BCG ip or id demonstrated significantly increased survival as did mice receiving BCG diluted 1:5 ip (Table I). Animals receiving BCG diluted 1:5 id were not protected.

Effect of HSV-2 antiserum. When 0.05 ml of high titered anti-HSV-2 rabbit serum was administered ip to newborn mice 24 hr after virus inoculation, a significant increase ($P < 0.01$) in survival rate as compared to controls was noted and the animals that succumbed died later than control animals (Fig. 3). This partial protective effect of immune serum is similar to that described previously (8). No significant difference in survival was

TABLE I. SURVIVAL OF NEWBORN MICE RECEIVING VARYING AMOUNTS OF BCG INTRAPERITONEALLY OR INTRADERMALLY 6 DAYS PRIOR TO CHALLENGE WITH $10LD_{50}$ OF HSV-2.

Treatment	Number of mice	
	Alive	Dead
None	0	9
Undil. BCG ip[a]	3	3
1:5 BCG ip[b]	11	9
Undil. BCG[c] id	5	2
1:5 BCG id	0	5

[a] $P < 0.05$ by exact test.
[b] $P < 0.005$ by exact test.
[c] $P < 0.0005$ by exact test.

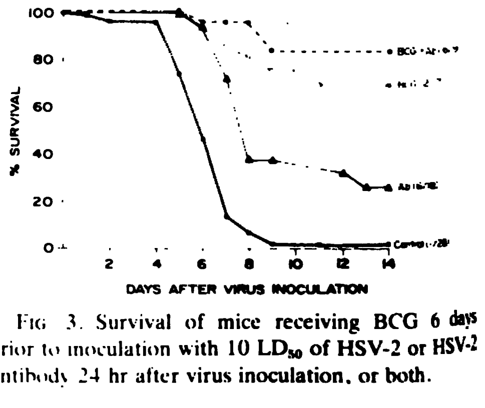

FIG. 3. Survival of mice receiving BCG 6 days prior to inoculation with 10 LD_{50} of HSV-2 or HSV-2 antibody 24 hr after virus inoculation, or both.

nice receiving BCG 6 days prior to
ulation, then antibody 24 hr later,
red to mice receiving BCG alone
challenged with virus ($P > 0.05$).
ion. Administration of adequate
BCG ip or id to newborn mice 6
r to ip challenge with HSV-2 re-
significantly increased survival. A
effect could not be demonstrated
G was given 4 days or less before
with HSV-2, suggesting that stim-
effective immune mechanisms re-
eral days. In this regard, Young
nes reported that it takes 5 days
sed numbers of lymphocytes and
ges to appear in the peritoneal
f newborn mice inoculated with
erium lepraemurium ip (9).

ctive effect was not obtained with
other agents used. It is possible
er doses or multiple administra-
ht have been effective. It is inter-
ote that of the agents used BCG is
ne to contain live organisms.

mechanisms might be involved in
ved increased resistance of new-
to HSV after administration of
rlier studies emphasized the role of
ges in immunity to HSV (4), and
ntly, activated macrophages have
vn *in vitro* to decrease HSV cell-to-
d (10). It is possible that BCG
macrophages which are then able
fection with HSV. The activation
phages might involve mediators
by T lymphocytes stimulated by
ernately, BCG might act by stimu-
duction of other lymphokines or of
te-dependent cytotoxicity.

ave been several reports on induc-
nmunity to HSV infections after
inistration, but the present report
t in which protection of newborn
demonstrated. Baker *et al.* (11)
rted a synergistic effect of HSV
erum and BCG on the survival of
e challenged with HSV-2 intrava-
lo protective effect could be dem-
when BCG was administered
r to viral challenge, a finding we
oborated in unpublished experi-
is group of workers had also re-
rlier on the partial protection conveyed by BCG on the mortality of adult rabbits infected with HSV-2 (12).

Favorable results have been claimed on the use of BCG for the treatment of recurrent genital herpetic infections (13), but the efficacy of this regimen requires further evaluation. Whether BCG might be helpful for the treatment or prevention of human neonatal HSV infections also remains to be ascertained. The studies reported here suggest, however, that newborns with already disseminated infection would be unlikely to benefit from BCG administration, since BCG was effective only if given 6 days prior to viral challenge. However, since the incubation period of HSV is around 6 days and it may take several days for disseminated disease to occur even after skin lesions are noted, BCG might affect the course of babies delivered vaginally from mothers with genital HSV infection at the time of delivery, or of newborns with herpetic skin lesions without signs of dissemination. The combination of BCG with other modalities of therapy, such as passively administered antibodies or antiviral agents, also remains to be determined.

A small, but significant, increase in survival rate was noted when high-titered anti-HSV-2 rabbit serum was administered to newborn mice 24 hr after virus inoculation (Fig. 3). Recently it has been shown that administration of similarly prepared antisera to newborn mice 1 hr after intranasal inoculation with HSV-2 results in increased survival (14). Since high-titered anti-HSV sera could conceivably be employed clinically, further studies on the use and activity of high-titered anti-HSV sera are justified.

Summary. Since age-dependent diminished macrophage function has been related to the increased susceptibility of newborn mice to herpes simplex virus (HSV) infection, the effect of several agents which might activate macrophages or other cells involved in immunity was investigated. BCG, typhoid vaccine, brucella vaccine, levamisole, or staphage lysate were administered to newborn mice prior to challenge with HSV-2. Of these agents, only BCG, administered ip or id 6 days prior to challenge, was found to increase the survival rate of newborn mice. The possible use of

BCG, alone or in combination with other modalities, for the prevention or treatment of neonatal HSV infections is discussed.

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vents in the Lytic Infection of Primary Mouse Kidney Cell Culture with Polyoma Virus. The Effect of Various Input Multiplicities (39328)

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(Introduced by R. A. Consigli)

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a (Py) virus, a papovavirus con-
cular double-stranded DNA, has
nsively studied in both the lytic
ve infection, in this and other lab-
Nevertheless, the precise se-
events occurring in early infection
ecise interaction of the virus with
mains yet to be elucidated.
Py-RNA is defined as the species
cific RNA which is found in lyti-
ted cells before the onset of viral
hesis, or in cells in which DNA
s blocked (5, 7, 10). In this re-
escribe the effect of various input
ies on the synthesis of early viral
data suggest that: (i) the rate of
hesis with respect to various input
ies is different early in infection
r at 27°) than it is later (i.e., 65
ii) the interaction of more than
particle with each cell appears to
d for the earlier appearance of
fic RNA.
s and methods. The preparation
s stocks and the primary mouse
l cultures (3, 10, 11), the modi-
dure for RNA-DNA hybridiza-
nd the technique for virus titration
ll previously been described. The
extracted by the hot phenol
, 10) and four "cycles" of ethanol
on. The RNA-DNA hybridiza-
erformed in 4 × SSC at 65° for

iral adsorption, the cells were
d at 27° (to slow the process of
in the presence of FdU (6 × 10^{-5}
the absence of serum (to block
hesis). The exact conditions and
teristics of infection under these
have also been described previ-
, 9). At the times indicated, the
s labeled with 250 μCi of
e (NEN, sp act 25 Ci/mmol) per ml of Dulbecco's reinforced Eagle's medium for a period of 3 hr, at which time the total cell RNA was extracted. The amount of RNA was estimated by measuring the absorbency at 260 μM in a Zeiss PM-2 spectrophotometer and the specific activity (cpm/μg RNA) was calculated. In most instances, 60 μg of RNA was used per hybridization, with DNA in excess.

Acid-soluble nucleotide pool determinations were performed essentially as described by Consigli and Ginsberg (4). Cells were infected at an m.o.i. of 20, or were "mock-infected," and were incubated at 27° for 65 hr in the presence of FdU. The medium was then replaced with fresh medium containing 0.5 μCi/ml of [^{14}C]uridine (NEN, sp act 50 mCi/mmol). After an additional 3-hr incubation, perchloric acid (PCA) extracts were prepared, concentrated by charcoal adsorption and elution, and hydrolyzed in formic acid at 175° for 45 min. After chromatography (isopropanol: HCl (conc):H_2O = 170:41:39), the spots were identified under a uv lamp, excised, and extracted with 0.1 *N* HCl. After uv spectrophotometry, aliquots were spotted on filter papers, dried, and the radioactivity was measured.

Results. Figure 1 shows the percentage of labeled RNA hybridizable to Py-DNA at times up to 65 hr after infection (27°, FdU) and input multiplicities ranging from 10^{-4} to 2×10^2 PFU per cell. At 27°, Py-RNA first becomes detectable at about 20 hr after infection. The amount of Py-RNA (percentage labeled RNA hybridizable to Py-DNA), synthesized at 65 hr after infection, approaches a linear relationship with respect to increasing m.o.i.s above 1. The amount of Py-RNA synthesized at lower m.o.i.s is consistent with that expected in the small proportion of cells which are infected. The relationship of the increasing m.o.i. to the

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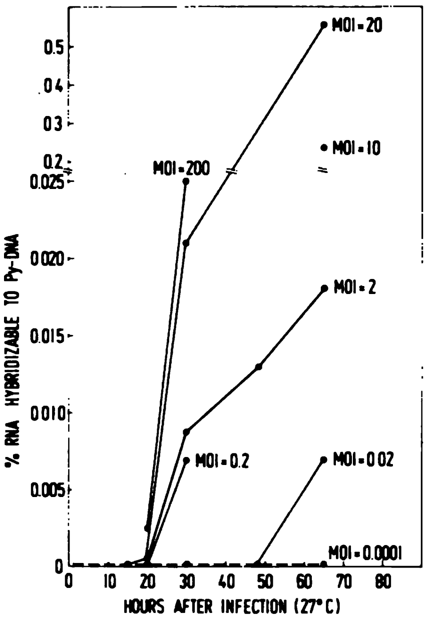

FIG. 1. The percentage of total RNA hybridizable to nitrocellulose filters containing an excess of Py-DNA is plotted for various multiplicities of infection. The infection was carried out at 27°, in the presence of 6 × 10^{-5} *M* FdU. At the times indicated, the cultures were "pulsed" for 3 hr (at 27°) with [^{3}H]uridine after which the RNA was extracted. The precise techniques have been described previously (see Materials and Methods). The radioactivity adhering to filters without DNA (blanks of 20–30 cpm) have been subtracted from the total counts per minute hybridized. One-tenth percent hybridizable RNA represents approx 2000 to 4000 cpm. Before plotting the data, the levels found in mock-infected cells (0.002–0.004%) have been subtracted from the values found on infection.

amount of Py-RNA synthesis at 30 hr is different than at 65 hr in that it appears that a maximum level is approached at the higher m.o.i.s studied.

"Early" in Py infection, there is no detectable change in the total amount of RNA present within the cells (unpublished data). There is a striking increase in the specific activity of RNA labeling with time after infection and with increasing m.o.i. (data not shown, see Fig. 2). The relationship between the increase in specific activity of RNA labeling and the logarithm of the amount of Py-RNA synthesized is shown in Fig. 2. To investigate the cause of this increase in RNA specific activity, the uracil pool size and rate of labeling was studied. Acid-soluble nucleotide pool determinations (3) were performed at 60 to 65 hr after infection (27°) following a 3-hr "pulse" with [^{14}C]uridine. The cells infected at an m.o.i. of 20, at 65 hr, contain 40% less PCA soluble uracil as compared with mock-infected cells. In addition, the infected cells incorporated 1.4 times as much labeled uridine from the medium. Both of these changes together lead to a sufficient increase in the specific activity of labeling of the uracil pool to account for the 2.1-fold increase in RNA labeling under the conditions cited (m.o.i. = 20 and T = 65 hr). A more detailed analysis of nucleotide pool changes will be forthcoming in a subsequent publication.

Discussion. At 27°, in the presence of FdU, Py virus infection at various input multiplicities, is associated with the synthesis of an amount of Py-specific RNA which is at m.o.i.s in which most or almost all the cells would be expected to be infected, more nearly quantitatively proportional to "gene-dose" at 65 hr than at 30 hr after infection. This may be due to the following: (i) the "saturation" of existing (viral) RNA-synthesizing capacity early with an increase of

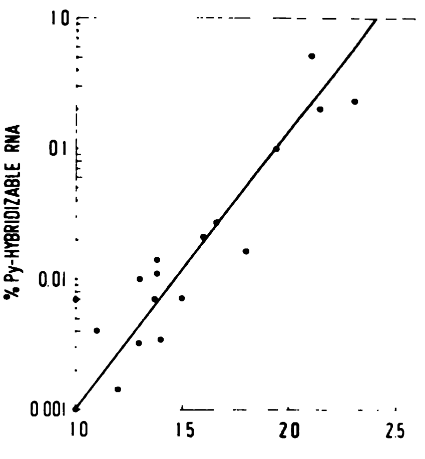

FIG. 2. The relationship between the amount of polyoma-specific RNA (see legend to Fig. 1) and the stimulation of relative specific activity of RNA labeling. (Cpm/μg RNA on infection − cpm/μg RNA of mock-infected cells.) Note the semilogarithmic coordinates which were used for convenience in plotting the data.

pacity later; and (ii) it is possible that te of uncoating of the adsorbed virus (therefore, its availability as template nscription) is limiting. More complex ations are also possible, but not prob- There appears to be a delay in the rance of Py-RNA when the m.o.i. from 0.2 to 0.02. These values are ated with a drop in the numbers of irus particles from approx one per cell s than one per cell. The DNA con- in these nonplaque-forming virus les may be less efficient templates for ription. The hypothesis that the DNA defective particles may serve as a ate for transcription is supported by inary experiments regarding the ki- of T-antigen production (as deter- by the proportion of nuclei stained a fluorescent antibody technique), we find is similar to that of early Py- with respect to the m.o.i.

changes in uracil pool size and the f uptake of uridine from the medium, obably responsible for the increase in ecific activity of RNA labeling, which n is related to early Py genetic expres- It is conceivable that some change in ll membrane affecting its permeability be responsible for this effect.

mary. Early polyoma (Py) virus-spe- NA synthesis was examined in cells ed with different concentrations of Py- The effect of various multiplicities of ion (m.o.i.) on the rate of Py-RNA sis is different at 30 hr as compared to . Thirty hours after infection at 27°, in resence of 5-fluoro-2-deoxyuridine), an increase in input multiplicity was not associated with a quantitatively commensurate increase in the amount of virus-specific RNA synthesized. At 65 hr, the amount of viral RNA synthesized was roughly proportional to the number of infecting virus particles.

I would like to thank R. Consigli, R. O'Brien, R. Bird, H. Turler, and M. Ho for their critical readings of the manuscript. This work was supported by the Swiss National Foundation for Scientific Research. A. Brown is the recipient of an American Cancer Society Fellowship, No. PF 791.

I would like to thank C. Salomon for technical assistance, and O. Jenni for preparing the figures.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 1

Ammonia Formation from Glutamine I
Perfused Rat Ki

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Montreal, Que

Glutamine-dependent renal ammonia production arises from a dual enzyme system, cytoplasmic D-glutamyltransferase (EC 2.3.2.1), and mitochondrial glutaminase I (EC 3.5.1.2), whose activities subserve different renal functions (1, 2). Besides being situated in separate cellular compartments (2), the two pathways can be differentiated by the fact that the mitochondrial glutaminase is highly stereospecific, acting only upon the L-isomer (3), while the cytoplasmic glutamyltransferase acts on either the L- or D-isomer (2, 4). I therefore employed these two isomers in order to demonstrate the relative activities of the two pathways in nonacidotic and chronically acidotic rat kidneys.

Materials and methods. Kidneys from nonacidotic and NH_4Cl-induced chronic acidotic (5) male Sprague-Dawley rats were isolated and perfused as previously described (6, 7). The perfusion solution consisted of an artificial plasma media with Dextran 110 in place of albumen providing glutamine as the sole source of exogenous nitrogen in these studies. Glutamine was employed at initial concentrations listed in the tables; the purity of commercially supplied (Sigma) D-glutamine was determined after exposure to *Escherichia coli* glutaminase and the liberated ammonia measured. With the five different lots supplied, apparent contamination by L-glutamine was always less than 4% (Sigma considers D-glutamine pure within the limits of their analysis). Perfusing kidneys with 0.04 m*M* L-glutamine, the maximal L-isomer present when perfusing with 1 m*M* D-glutamine, failed to increase ammonia production above those levels observed in the absence of exogenous glutamine. Perfusate ammonia concentrations were measured at 15-min intervals throughout the 60-min perfusion period using a modification of the glutamate dehydrogenase NADH-NAD linked reaction

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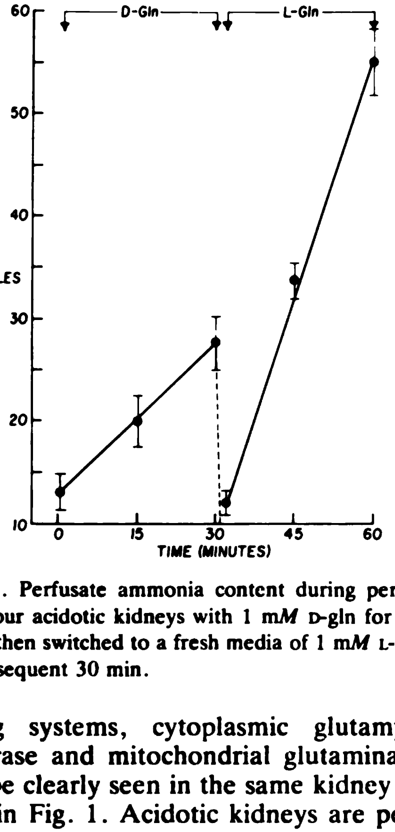
TABLE I. AMMONIA PRODUCTION FROM GLUTAMINE ISOMERS BY NONACIDOTIC RAT KIDNEYS.

utamine concentrations (mM)	Ammonia production (μmole hr^{-1})	Glutamine uptake (μmole hr^{-1})	Ammonia / Glutamine
D-Gln			
1 (4)[a]	16.2[b] ± 3.6	16.8 ± 4.3	0.96
2.5 (4)	21.6 ± 4.2	27.0 ± 5.7	0.80
5.0 (4)	37.8 ± 6.9	44.3 ± 7.5	0.85
L-Gln			
1 (4)	20.4 ± 2.0	15.0 ± 3.8	1.36
2.5 (4)	27.6 ± 4.6	23.4 ± 5.1	1.18
5.0 (5)	36.6 ± 8.4	37.8 ± 6.6	0.97

Numbers in parentheses represent perfused kidneys.
Mean ± SE.



IG. 1. Perfusate ammonia content during perfu- of four acidotic kidneys with 1 mM D-gln for 30 and then switched to a fresh media of 1 mM L-gln i subsequent 30 min.

zing systems, cytoplasmic glutamyl- isferase and mitochondrial glutaminase in be clearly seen in the same kidney as wn in Fig. 1. Acidotic kidneys are per- d with D-glutamine which penetrates the ochondrial inner membrane in acidosis but because of specificity restrictions, not be hydrolyzed by matrix-located glu- inase I, and as a consequence ammonia contributed solely by the cytoplasmic way. After 30 min the perfusion media vitched, by means of a 3-way stopcock, i fresh perfusion solution containing L- amine and ammonia production then is wed for a subsequent 30 min. Switching i D- to L-glutamine reveals the striking duality of the cytoplasmic and mitochondrial pathways in the acidotic kidney. Ammonia production by the cytoplasmic pathway increased about twofold 16.2 μmole hr^{-1} (Table I) to 29.4 μmole hr^{-1} (Table II), a rise in activity which is consistent with previous observations (9–11).

Ammonia production by the mitochondrial pathway rose from 4.2 μmole hr^{-1} (the difference between L- and D-glutamine's ammonia production, Table I) to 84.7 μmole hr^{-1} (Table II) giving an amazing 20-fold activation as previously observed (1, 12). Apparently, under these conditions, the mitochondrial pathway successfully competes for L-glutamine since the NH_3/gln ratio of 2.0 indicates complete deamidation and deamination of glutamine (13–15). These are the highest ratios I have observed and are probably the result of depriving this pathway of substrate during the perfusion with D-glutamine and the elevated glutaminase I-glutamine utilization rate.

Discussion. The purpose of this report was to demonstrate the existence of the glutamyltransferase and glutaminase I-gluta-

TABLE II. AMMONIA PRODUCTION BY ACIDOTIC KIDNEYS PERFUSED WITH D-GLUTAMINE (0–30 MIN) AND L-GLUTAMINE (32–62 MIN).

Glutamine concentration (mm)	Ammonia production (μmole hr^{-1})	Glutamine uptake (μmole hr^{-1})	Ammonia / Glutamine
D-Gln			
1 (4)[a]	29.4 ± 4.8	30.6 ± 3.6	0.96 ± 0.06
L-Gln			
1 (4)	84.7 ± 6.6	42.5 ± 2.4	1.99 ± 0.06

[a] Numbers in parentheses represent perfused kidneys.

mine utilizing pathways from the perspective of substrate specificity. In addition, the quantitative contribution of each pathway to the total renal ammonia production can be estimated in the normal and chronically acidotic kidneys. The present studies, in agreement with others (1, 2, 10), support the interpretation that glutamyltransferase is the major producer of ammonia in the normal acid-base state, while a 15- to 20-fold activation of the mitochondrial pathway (1, 12) makes it the predominant source of ammonia in acidosis as previously observed (16, 17). The existence of dual pathways has been substantiated from the perspective of nitrogen (1, 7) and carbon (1, in preparation) end product analysis, selective inhibition of one, glutamyltransferase (2, 9, 10), or the other, glutaminase I (11), and by stimulating preferentially one pathway, glutaminase I in acidosis (16), or the other, glutamyltransferase by amino acid loading (18). All of these diverse experimental approaches have supported the major conclusion of the present study.

Summary. The following points summarize these findings: (i) there are 2 glutamine utilizing enzyme systems in the rat kidney; (ii) the cytoplasmic glutamyltransferase system hydrolyzes either glutamine isomer while the mitochondrial localized glutaminase 1 is specific for the L-isomer; (iii) the cytoplasmic pathway contributes 70% of the total renal ammonia production in the normal kidney; (iv) chronic metabolic acidosis results in a 20-fold activation of the mitochondrial glutaminase 1 pathway.

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patic Carbamyl Phosphate Synthetase and Ornithine Transcarbamylase in Mouse Influenza A and Influenza B Infection[1] (39330)

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e's syndrome, an acute encephalop-
ccompanied by alterations in liver
re and function has been recognized
umber of years as an infrequent com-
on of varicella-zoster infection, mea-
nfluenza, and other virus infections
Neurological and hepatic abnormali-
ere also described in an influenza A
nic in India in 1958 by Kapila (5).
tly, however, Reye's syndrome has
nost prominently associated with in-
a B infection, and there are only scat-
reports of its occurrence after influ-
infection (4, 6–8).

ically, the syndrome develops ab-
after partial recovery from the pro-
l virus infection that is usually not se-
t is characterized by high fever, nau-
d vomiting, abdominal pain with rapid
of cough, seizures, coma, and fre-
y death. Despite severe cerebral dys-
n with increased spinal fluid pres-
erebrospinal fluid cell counts and pro-
e usually not increased (1, 2).

etiology of the disease is obscure, but
ars that the encephalopathy may re-
om ammonia and possibly other me-
es of the urea cycle that accumulate in
d concentrations in blood and tissue
e of impaired function of one or more
ycle enzymes in liver (9, 10). Thaler
s associates (10) found that activity of
epatic mitochondrial enzyme, orni-
transcarbamylase (OTC), was only
of normal in a patient with Reye's
me, and that there was an 18-fold
ion in the affinity of this enzyme for
ine. Brown *et al.* (11) observed a re-
n in carbamyl phosphate synthetase (CPS) as well as OTC in patients with Reye's syndrome. Tang and his associates (9) report a reduction to 10% of normal OTC activity in a patient with Reye's syndrome. The suggestion was made that Reye's syndrome may occur in persons having a genetically determined deficiency of an enzyme(s) of ammonia metabolism that is triggered by a variety of acute virus infections (10).

The apparent more frequent occurrence of Reye's syndrome with influenza B infection than with influenza A suggested the possibility, however, that some circumstance associated with influenza B infection might interfere with the activity of one or more urea cycle enzymes, resulting in hyperammonemia. This possibility was emphasized by the fact that influenza A occurs more frequently and is more severe than influenza B. Therefore, the hepatic levels of CPS and OTC were determined in mice during experimental infection with the two types of influenza.

Materials and methods. Virus. Experimental infection was produced by 0.05-ml intranasal instillation of a suspension of virus in mice anesthetized with ether. Control mice were treated with intranasal instillation of 0.85% saline following ether anesthesia. Influenza A/PR/8/34 (HONI), Ferret 8, Mouse 593, Egg 170, Mouse 1, Egg 1 ($10^{7.5}$ $TCID_{50}$/0.2 ml, rhesus monkey kidney cell culture) was used in a dilution of 1:10,000, and influenza B/Lee/40, Ferret 8, Mouse 137, Ferret 127, Mouse 23, Egg 1 (10^5 $TCID_{50}$/0.2 ml, rhesus monkey kidney cell culture) in a 1:100 dilution. Both virus strains were obtained from Dr. Walter Dowdle, Center for Disease Control, Atlanta, Georgia. Subsequent passages of both viruses were reidentified by hemagglutination inhibition with antiserum provided by CDC and found to be authentic.

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served.

Mice. Female white Swiss mice (CDI type) weighing approx 20 g were obtained from Charles River Breeding Farms. Neonatal mice were obtained from Microbiological Associates, Washington, D.C. For liver enzyme assays, mice were sacrificed on Day 5 of influenza A infection, and on Day 6 with influenza B infection. A few mice with influenza A infection died spontaneously before the time of sacrifice and were included. Assays of livers from these animals were not appreciably different from those of sacrificed animals. Livers were weighed, visually inspected, and frozen in Dry Ice immediately after autopsy. The lungs of all mice were examined for gross pathology at the time of sacrifice or death.

Human liver was obtained within 8 hr of death from six patients, none of whom had liver disease. Their age, sex, and diagnoses were as follows:

Age	Sex	Diagnosis
49	F	Cerebral hemorrhage
49	F	Left midcerebral arterial occlusion, hypertension
20	M	Accident
52	M	Coronary artery occlusion
50	M	Gun shot wound—head
46	M	Cerebral hemorrhage

Enzyme assays. The whole livers were kept frozen at −70° until immediately before assay. No appreciable differences in the levels of the enzymes measured occurred due to the freezing procedure. The livers (approx 1.0 g) were suspended individually in 19 ml of 4° water and homogenized using a Ten Broeck tissue grinder (10 strokes). The homogenate was centrifuged for 5 min at 600 rpm to remove unbroken cells and cell debris. The supernatant was used as the enzyme preparation.

CPS. The substrate mixture (12) contained (μmole/ml): NH_4HCO_3, 100; ATP, 10; L-ornithine, 10; *N*-acetyl-L-glutamate, 10; and $MgSO_4$, 20. The substrate solution was stored at −70° to prevent deterioration. Prior to use, the substrate mixture was saturated with CO_2. The reaction mixture consisted of 0.40 ml of the substrate mixture (preincubated at 37° for 3 min), 0.25 ml of 50 m*M* NH_4HCO_3 (containing 100 units of OTC-bovine), and 0.15 ml of the liver homogenate was added to initiate the reaction. The complete reaction mixture was allowed to incubate at 37° for 15 min, at which time the reaction was terminated by the addition of 0.2 ml of 15% perchloric acid. The resulting precipitate was removed by centrifugation (4000 rpm for 5 min). A 0.1-ml aliquot of the supernatant was used for the determination of citrulline as described by Hunninghake and Grisolia (13).

OTC. The reaction mixture contained (μmole/ml): carbamyl phosphate, 20; L-ornithine, 15; NH_4HCO_3 (pH 7.8), 50; and 0.01 ml of liver homogenate in a total volume of 0.8 ml. The reaction was initiated by the addition of the homogenate to the preincubated (3 min at 37°) substrate mixture. The reaction mixture was incubated at 37° for 15 min and then terminated by the addition of 1.2 ml of 15% perchloric acid. The mixture was centrifuged for 5 min at 4000 rpm, and 0.06 ml of the supernatant was utilized for citrulline determination.

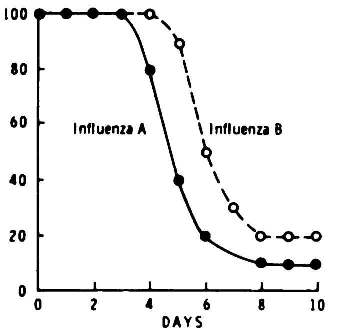
It was determined for both CPS and OTC that product formation was linear over the time interval utilized and for the concentrations of enzymes and substrates used in the reaction mixtures. The protein concentration was estimated by the method of Lowry *et al.* (14) and specific activity is expressed as micromoles of citrulline produced per minute per milligram of protein. Carbamyl phosphate (dilithium salt) and L-ornithine were purchased from Sigma Chemical Company and all other chemicals were of highest purity commercially available. The bovine OTC was provided by Dr. Steve Bishop (Department of Biochemistry, Baylor College of Medicine).

Results. Characteristics of influenza A and B infection in the mouse. In Fig. 1 is shown the mortality of two groups of 10 mice following intranasal inoculation with influenza A/PR/8/34 (HONI) or influenza B/Lee/40. Deaths from influenza A infection began on Day 4 and by Day 8, 9 of 10 mice were dead. Deaths from influenza B infection began on Day 5 and by Day 8, 8 of 10 mice had died. In Fig. 2A is shown daily food consumption of the mice described in Fig. 1, with sham-inoculated uninfected mice serving as controls. In mice infected with influenza A, food consumption dropped progressively from 4 g to 0.25 g per day per mouse by Day 5, while food consumption of mice vith influenza B dropped to about day in this period of time.

2B is shown the daily weight of ing infection with influenza A and g influenza A infection, the aver- ht dropped from 20 g to 15 g by hile with influenza B infection it to 18 g. Uninfected mice gained veraging 22 g by Day 6.

ngs of all mice were examined for hology. Pneumonia was extensive all animals and was most severe in culated with influenza A.

d OTC activity in livers of infected natal mice, and in man. Values for iepatic enzymes in adult mice in- th influenza A and B are shown in Only in mice infected with influ- ere levels of enzyme activity signif- duced. A 12 and 17% reduction rved in CPS and OTC activities, ely. Intraperitoneal or intravenous ation of the influenza A or influ- d not result in a fatal disease, and



Mortality from influenza A and influenza B e Methods). There were 10 mice in each

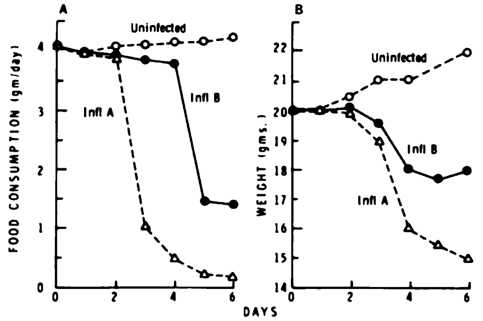


FIG. 2. (A) Reduction in food consumption in mice infected with influenza A and B infection. Values represent mean consumption by mice surviving each day. There were two cages of five mice at the start of the experiment. (B) Reduction in body weight during course of infection with influenza A and B.

CPS and OTC activities were unaltered.

Livers from 10 neonatal mice were also assayed (Table I) for the activity of these enzymes. CPS values were about 60% of the level of young adult mice (controls in Table I) while OTC values in neonates were 40% of those in normal adult mice. There was a 5–10% reduction in both CPS and OTC activities in neonatal mouse livers during influenza A or influenza B infections (not shown). For purposes of comparison, assays for CPS in livers obtained at autopsy from five adult human patients without known liver disease were 1.6 times greater than those in adult mice. However, OTC values in the human livers were only 30% of the values for adult mice. The values for both enzymes in livers of human adults were comparable to most published values for human neonates and adults (10, 15–19).

Discussion. The principal finding of this

ABLE I. SPECIFIC ACTIVITY OF HEPATIC CARBAMYLPHOSPHATE SYNTHETASE AND ORNITHINE ARBAMYLASE IN ADULT MICE INFECTED WITH INFLUENZA A AND B, IN NEONATAL MICE, AND IN HUMAN ADULTS WITHOUT LIVER DISEASE.[a]

	Number	Carbamylphosphate synthetase ($\times 10^2$)	Ornithine transcarbamylase
lt Mice			
ontrols	20	3.03 ± 0.21	1.90 ± 0.09
fluenza A	20	2.66 ± 0.13	1.58 ± 0.20
fluenza B	20	2.97 ± 0.20	1.79 ± 0.23
natal mice	10	1.77 ± 0.16	0.718 ± 0.25
nan adults	6	5.02 ± 2.37	0.568 ± 0.166

activity is expressed as micromoles of product produced per minute per milligram of protein. Mice ated (intranasal) with either saline (control) or influenza A or B as indicated.

l significance: One-tailed t-test, 38 *df*. CPS, control vs influenza A, $t = 1.96$, $P < 0.05$. OTC, control A, $t = 2.76$, $P < 0.005$. No other pairs involving infected mice were significantly different.

study is that highly lethal infections in mice following intranasal inoculation with influenza A or influenza B were associated with only a moderate reduction in activity of two hepatic enzymes of the urea cycle, CPS and OTC. The activity of the two enzymes in both infections ranged from 83 to 98% of normal. Since the CPS and OTC levels of neonatal mice were intrinsically lower than adult levels, it was thought that small reductions in CPS and/or OTC activities could induce hyperammonemia. However, CPS and OTC activities of neonatal mice infected with influenza resulted in no more than a 10% reduction of either enzymatic activity. In the few human patients with Reye's syndrome in whom these measurements have been made, enzyme activity has been in the range of 10 to 20% of normal. The reductions of enzyme activity in the present study are, therefore, probably a nonspecific result of the debilitating effect of illness.

Thus, influenza infection in mice does not result in a sufficient reduction in CPS or OTC activities to produce hyperammonemia. If the foregoing thesis is correct, then the search for the pathogenetic mechanism of the syndrome must turn toward factors related to the patients or features of the epidemiology of the various diseases that predispose to development of Reye's syndrome. The injury from virus infection responsible for reduced enzyme activity may result directly from a dissemination of virus to the viscera including the liver (4, 5, 9), despite an apparent mildness of the prodromal illness. Other hepatic enzymes and functions are also affected in Reye's syndrome, and the relationship among these various alterations cannot be clearly defined at present.

From an epidemiological point of view, the apparent paradox of relative absence of Reye's syndrome with influenza A and its more frequent presence with influenza B attracts attention, but the present study did not afford an explanation for this difference.

Summary. In mice infected with mouse-adapted influenza A/PR/8/34, hepatic carbamyl phosphate synthetase (CPS) activity was reduced to 88%, and ornithine transcarbamylase (OTC) was reduced to 83% of control values. In mice infected with mouse-adapted B/Lee/40, CPS activity was 98% and OTC was 94% of control values. These limited reductions in enzyme activity were attributed to a nonspecific debilitating effect of acute influenzal pneumonia. These findings suggest that the pronounced reduction of CPS and OTC activities reported in Reye's syndrome in man are not a general manifestation of the severity of influenza infection.

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ed Implantation Caused by Administration of Sheep Immunogamma Globulin against LHRH in the Rat[1] (39331)

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indispensability of the pituitary gland maintenance of early pregnancy in was documented as early as 1933 by arz and Long (1). In the same year, t *al.* (2) also reported that pituitary otropin plays an important role in the alf of pregnancy in the rat, since hy- sectomy before Day 11 of pregnancy ated pregnancy. Pregnancy of rats hy- sectomized before Day 7 was main- by gonadotropin(s) combined with tin (3–5) or prolactin combined with en (5, 6). More recent studies using um to LH have shown that antiserum administered prior to Day 8 of preg- delayed implantation (7, 8) or caused on in the rat (8–11). Delayed implan- resulting from antiserum to LH was d by administration of estrogen (12), ting that the antiserum which may eutralized the activity of endogenous used a reduction of estrogen secre-

well established that the secretion of d probably FSH as well is regulated RH. If the secretion of gonadotropins early pregnancy is controlled by the alamus by means of LHRH, neutrali- of the activity of endogenous LHRH specific antiserum to LHRH would early pregnancy. The information on ect of administration of anti-LHRH on pregnancy would shed light on the f the hypothalamus in establishing ncy. The present study deals with the of sheep anti-LHRH gamma globulin lantation of fertilized ova in the rat.

ods and materials. Adult rats of Charles River, CD strain were used throughout the experiment. They were maintained in an animal quarter artificially illuminated from 0500 to 1900 hr daily. The animals were given free access to Purina Laboratory chow and water. One week after arrival, vaginal smears were checked in the female rats every morning as described by Everett *et al.* (13), and only rats exhibiting at least two consecutive 4-day cycles were selected for this study. On the afternoon of proestrus (P), one female rat was caged with two male rats which had been proven fertile. The next morning, the vagina of the female rat was inspected for the presence of spermatozoa by vaginal lavage with 0.9% saline. If spermatozoa were present, that day was designated as pregnancy Day 1.

One milliliter of sheep anti-LHRH gamma globulin (anti-LHRH-G) or normal sheep gamma globulin (NSG) was injected into the jugular vein of the rat under ether anesthesia between 0900 and 1000 hr on the day indicated in the Results.

Blood was collected from the jugular vein of some of these animals on various days during the pregnancy. All animals underwent laparotomy under Nembutal anesthesia (3.5 mg/100 g BW) on Days 8 and 14 for inspection of implantation sites. Some of the animals were allowed to survive to term.

Antiserum to LHRH was generated in two ewes by immunizing the animals with multiple injections of Glu^1-LHRH (Dr. D. H. Coy) which was conjugated with human serum albumin (HSA). Three-and-one-half milligrams of HSA-Glu^1-LHRH conjugate was injected into each ewe at 2-week intervals as described elsewhere (14). One of these ewes (No. 772) produced the antibody to LHRH with a singificant titer after the fifth immunization.

Anti-LHRH-G was isolated from the antiserum by ammonium sulfate by two-step precipitation procedures (15), then dis-

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quests for reprints should be addressed to: Dr. rimura, VA Hospital, 1601 Perdido St., New , Louisiana 70146.

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solved in 0.9% saline and dialyzed against 0.9% saline for 72 hr at 4° to remove ammonium sulfate. The dialyzed gamma globulin was adjusted for volume by adding 0.9% saline to make up the original serum volume. Ten-milliliter aliquots were placed in vials, lyophilized, and stored at 4°. Shortly before the experiment, the content of each vial was dissolved in 10 ml of H_2O. Gamma globulin from normal sheep serum was similarly prepared.

Antigenic determinants of the anti-LHRH-G were determined by examining the cross reactivity with various synthetic peptides corresponding to fragments of LHRH decapeptide, as described elsewhere (16). Those peptides shown in Table I were kindly supplied by Dr. Yanaihara.

Serum samples collected from rats at various stages of pregnancy were determined for LH and prolactin by radioimmunoassay (RIA). Serum LH was determined in duplicate by RIA as described by Niswender *et al.* (17), (OO-Rat-LH RIA) using NIH-LH-S 17 as the reference standard. Serum prolactin was measured in duplicate by RIA as described by Niswender *et al.* (18) using NIAMDD-RAT-Prolactin kit. The assay data of LH and prolactin were processed by a computer program for RIA described by Duddleson *et al.* (19). Mean serum hormone levels of each group were compared with those of others by using Duncan's new multiple range test. Synthetic LHRH preparation used (TAP 023) was kindly provided by Dr. M. Fujino, Takeda Chemical Industries. 17-β Estradiol was obtained from Schering Corp. (N.J.).

Results. Characterization of sheep anti-LHRH-G. Sheep anti-LHRH-G (no. 772) bound 99% of ^{125}I-LHRH at 1:70 dilution under the conditions of radioimmunoassay for LHRH (14). The antibody-tracer binding was inhibited by unlabeled LHRH in a dose-related manner, enabling us to establish a linear standard curve using logit of B/Bo% on the ordinate and log dose on the abscissa in a range from 16 to 4096 pg/tube, where B and Bo represented bound radioactivity with and without the presence of unlabeled LHRH. The antibody-tracer binding was not affected by other hypothalamic and pituitary hormones, including TRH, somatostatin, rat LH, FSH, and prolactin, and the extracts of the rat placenta. However, the binding was inhibited to various degrees by synthetic peptides corresponding to fragments of LHRH or LHRH analogs. As shown in Table I, only peptides which contained Leu-Arg-Pro-Gly-NH_2 showed significant cross-reactivity. Therefore, the antigenic determinant at the antibody side is considered to reside in this amino acid sequence.

Effect of anti-LHRH-G on implantation. The number of implantation sites on Days 8 and 14 of pregnancy in rats injected with anti-LHRH-G or NSG are summarized in Table II. Fourteen implantation sites on the average were clearly observed on Day 8 in the rats given NSG from Days 1 through 7 (Group 1). On Day 14, a similar number of

TABLE I. CROSS-REACTIVITY OF SHEEP ANTI-LHRH GAMMA GLOBULIN (NO. 772) WITH VARIOUS SYNTHETIC PEPTIDES CORRESPONDING TO FRAGMENTS OF LHRH DECAPEPTIDE.

Peptide.	Amino acid sequence	Cross-reactivity, %
LHRH	*p*Glu·His·Trp·Ser·Tyr·Gly·Leu·Arg·Pro·Gly·NH_2	100
1	*p*Glu ——— Leu·OH	0
2	*p*Glu ——— Gly·OH	0
3	*p*Glu —— Trp·OH	0
4	His ——— Leu·OH	0
5	Ser ——— Leu·OH	0
6	Leu ——— Gly·NH_2	30
7	Ser ——— Gly·NH_2	70
8	*p*Glu( )——— Gly·NH_2	100
9	*p*Glu( )——— Gly·NH_2	100
10	His ——— Gly·NH_2	100
11	*p*Glu ——— Gly·OH	0
12	Trp ——— Pro·OH	0
13	Trp ——— Gly·NH_2	100
14	Trp ——— Arg·NH_2	0

TABLE II. EFFECT OF ANTI-LHRH-G[a] ON IMPLANTATION IN RATS.

Group	Number of rats	Treatment	Duration of treatment	Mean implantation sites (Number of rats with implantation sites) Day 8	Day 14	Remarks
1	5	NSG[b]	Days 1-7	14 (5)	13 (5)	Normal parturition
2	8	Anti-LHRH-G	Days 1-7	0 (0)	5 (3)	Two delivered pups 7-8 days after term
3	6	NSG	Days 3-5	13 (6)	13 (6)	Normal parturition
4	12	Anti-LHRH-G	Days 3-5	0 (0)	13 (9)	Parturition delayed by 4-6 days
5	5	Anti-LHRH-G	3	13 (5)	14 (5)	Normal embryonic swellings on Day 14; normal parturition
6	6	Anti-LHRH-G	4	5 (1)	9 (4)	Parturition delayed by 3-4 days
7	6	Anti-LHRH-G	5	13 (6)	13 (6)	Some fetuses small on day 14; normal parturition
8	6	Anti-LHRH-G + 2 × 1 μg of LHRH/16% gelatine	4	13 (6)	12 (6)	Normal embryonic swellings on Day 14
9	5	Anti-LHRH-G + 1 μg of estradiol	4	10 (5)	10 (5)	Nearly normal, some small on Days 8 and 14
10	5	Anti-LHRH-G + 0.02 μg of estradiol	4	12 (4)	14 (4)	Most small on Day 14
11	6	Anti-LHRH-G + 0.01 μg of estradiol	4	12 (1)	14 (6)	Indistinguishable on Day 8, small on Day 14

[a] Sheep anti-LHRH gamma globulin.
[b] Normal sheep gamma globulin.

viable fetuses were present, and the animals delivered pups on term. When rats were injected with 1 ml of anti-LHRH-G once daily from Days 1 through 7 of pregnancy (Group 2), no implantation site was recognized on Day 8. On Day 14, three out of eight rats had viable fetuses but they were smaller than those found in NSG-treated control rats (Group 1). Two of these rats delivered pups but the parturition was delayed by 7-8 days.

Other groups were treated with anti-LHRH-G (Group 4) or NSG (Group 3) from Days 3 through 5. On Day 8, no implantation site could be distinguished in the rats treated with anti-LHRH-G, but in 9 out of 12 rats, embryonic swelling became evident on Day 14. These rats delivered pups 4-6 days later than term. The results indicate that treatment with 1 ml of anti-LHRH-G from Days 3 through 5 delayed mplantation by about 5 days but did not erminate gestation.

To pinpoint the critical time at which LHRH is essential for timely implantation, 1 ml of LHRH was injected iv on Day 3, 4, or 5 of pregnancy. A clearcut inhibition of implantation on Day 8 was observed when anti-LHRH-G was injected on Day 4, but no effect was obtained when anti-LHRH-G was administered on Day 3 or 5. In the rats treated on Day 4, implantation appeared to have been delayed by 3 days, judging from the day of parturition.

Prevention of delayed implantation by LHRH or estrogen. On Day 4, the rats of group 8 received one iv dose of anti-LHRH-G and two sc doses of LHRH in 16% gelatin/0.9% saline. Implantation sites on Day 8 were nearly normal (Table II). On Day 4 of pregnancy, different doses of estradiol were also injected together with anti-LHRH-G. A dose of 0.02 μg of estradiol injected concomitantly with anti-LHRH-G only slightly restored the delayed implantation. A nearly complete nullification of the inhibition effect of anti-LHRH-G on implantation was achieved by 1 μg of estradiol. These results suggest that delayed implantation by anti-LHRH-G is caused by neutralization of endogenous LHRH and, in turn, by the decline of estrogen secretion produced by this treatment.

Plasma LH levels on Day 4 or 5 appeared

to be lower after anti-LHRH-G injection. However, since the preinjection levels were in a range close to the margin of assay sensitivity (from nondetectable to 1.9 ng/ml), quantitative measurement of the reduction of plasma LH was impossible to make. There was no significant difference in plasma prolactin levels between groups treated with NSG and those treated with anti-LHRH-G.

Discussion. The results of the present study clearly indicate that hypothalamic LHRH is indispensable on Day 4 of pregnancy in rats for the timely implantation of fertilized ova, most probably by maintaining secretion of LH and, in turn, estrogen. Our studies indicate that serum LH in castrated rats declines as early as 2 hr after iv injection of sheep anti-LHRH serum (Vilchez-Martinez *et al.*, unpublished observation) and that injection of the anti-LHRH serum to proestrous hamsters at noon blocks midafternoon elevation of serum LH (20). These findings indicate that the blockade of the action of endogenous LHRH by exogenously administered antibody occurred within a few hours after injection. The fact that anti-LHRH-G could delay implantation only when injected on Day 4 of pregnancy, not on Day 3 or 5, suggests that Day 4 of pregnancy is the critical period when the hypothalamic hormone is most needed for a successful implantation. This also suggests that the effect of anti-LHRH-G does not last longer than 1 day.

Although we could not determine serum estrogen, the restoration by estradiol of the delay in implantation induced by anti-LHRH-G indicates that secretion of estrogen which is essential for implantation was suppressed below the threshold required for nidation. Although estrogen appears to play a major role in implantation, the function of progesterone, together with estrogen, in the process of implantation on Day 4 of pregnancy cannot be excluded. Plasma progesterone levels show two peaks during pregnancy in rats: the first small peak appears on Days 3-5 and a large peak on Days 13-20, implying a physiological significance at these periods (21). Plasma progesterone levels during early pregnancy are indeed decreased by the administration of anti-LHRH-G (22).

Daily injections of anti-LHRH-G from days 1 through 7 of pregnancy not only inhibited implantation on Day 8, but also on Day 16 in some animals. In some rats, resorption of fetuses was observed at the second laparotomy. This could be due to decreased secretion of progesterone which is essential for viability of the implanted fetuses (7, 21).

Loewit and Laurence (23) and Madhwa Raj and Moudgal (24) reported that administration of anti-LH serum to rats from Days 7 through 14 of pregnancy prevented or terminated gestation. Progesterone, however, given concomitantly with the antiserum to LH, overcame the inhibitory effect of anti-LH, and the pregnancy was maintained.

The results of the present study on antisera to LHRH resemble the findings obtained by using a minimum effective dose of anti-serum to LH which caused delayed implantation, but which did not terminate gestation (12). Madhwa Raj *et al.* (12) reported that the antiserum given to rats on the morning of Day 4 of pregnancy resulted in an inhibiton of implantation on Day 8 and that an LH surge precedes an estrogen surge on Day 4 of pregnancy. On the other hand, Morishige *et al.* (25) and Linkie and Niswender (26) reported elevated serum LH levels during early pregnancy in rats, from Days 1 through 11 and Days 1 through 4, respectively, but they did not find a significant LH surge on the afternoon of Day 4 of pregnancy. In the present study we could not observe clear-cut suppression of serum LH by anti-LHRH due to the limited sensitivity of the assay. However, our findings imply that the maintenance of basal secretion of LH during early pregnancy requires LHRH. It is possible that administration of antiserum reduces serum LH below the threshold levels for implantation.

When our findings on the effect of anti-LHRH-G are compared with those of others who have examined the effect of antiserum to LH, it appears that the effect of anti-LHRH-G is less pronounced than that of anti-LH serum. LH in circulation may be abruptly and nearly completely neutralized by the excess dose of antiserum, resulting in a sudden disappearance of the hormone. On the other hand, LHRH which is mainly con-to the median eminence and hypo- l portal blood may be subjected to the lizing action of exogenously adminis- anti-LHRH-G. This reduction of cir- g LH by anti-LHRH may take place gradually and less drastically than that l by anti-LH serum, and the circulat- I would never fall to zero because of sidual function of pituitary gonado- . Absence of vaginal bleeding in all of ts given anti-LHRH-G in the present could support this speculation.

mary. The effect of the administra- f sheep anti-LHRH gamma globulin HRH-G) on implantation of fertil- va was investigated in rats. Daily in- ns of 1 ml of anti-LHRH-G from Days ough 7 of pregnancy uniformly in- l implantation of fertilized ova on , but viable sites, though considerably r in size than in control rats, became guishable on Day 14 in most rats. In of these rats resorption of fetuses oc- , and others delivered pups 7–8 days erm. When the rats were given anti- I-G from Days 3 through 5, the im- tion was delayed by 5 days, but the on was not terminated. A single in- n of 1 ml of anti-LHRH-G on Day 4 ed implantation on Day 8, but injec- n Day 3 or 5 did not. The delayed ntation by anti-LHRH-G injected on was nullified by concomitant adminis- n of 2 sc injections of 1 μg of LHRH, ingle dose of 1 μg of estradiol. The ndicate that the hypothalamic LHRH ntial on Day 4 of pregnancy for timely ntation of fertilized ova, probably by ining LH and, consequently, estro- cretion.

authors thank Mrs. J. Gauthier and Mrs. D. for their technical help; Miss G. Farley for her preparing the manuscript; Dr. G. D. Niswender ntiserum to ovine LH, No. 15; NIAMDD Rat Hormones Distribution Program for reagents ; Dr. M. Fujino for LHRH; and Dr. N. Yanai- peptides corresponding to LHRH fragments.

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Release into Culture Medium of Membrane-Associated, Tumor-Specific Antigen by B-16 Melanoma Cells[1] (39332)

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Soluble tumor antigen, in a free or antibody-complexed form, has been implicated in the blockade of cell-mediated tumor rejection (1-4). We have recently reported the deposition of soluble immune complexes consisting of soluble tumor antigen and antibody, in the kidneys of mice bearing a progressive B-16 melanoma (3). The intensity of renal deposition correlated directly with tumor size and the presence of metastases, and inversely with the degree of mononuclear leukocyte infiltration of the tumor *in vivo*. It was of interest, therefore, to determine by what means and in what form the soluble antigen was generated by the tumor cells.

Soluble materials which exhibit tumor-associated antigenicity have been described recently in the expended medium of cultured L-1117 mouse lymphoma cells (5) and B-16 melanoma cells (6). We have described the occurrence of such antigens in the serum of B-16 bearing mice (3), and the present study has confirmed the presence of soluble tumor antigens in expended B-16 culture fluids.

Materials and methods. Preparation of soluble antigen from culture supernatant fluids. Monolayers of B-16 cells were cultured until they reached maximum density in RPMI-1640 medium containing penicillin (100 units/ml)-streptomycin (100 μg/ml), tylocine (anti-PPLO), and 15% heat-inactivated, virus-screened fetal calf serum (Grand Island Biological Company, Grand Island, New York) at 37° in a 5% CO_2 atmosphere. Monolayers were washed thoroughly with Hanks Balanced Salt Solution and cultured for 72-96 hr in serum-free RPMI-1640 medium as before. Supernatant medium was then decanted, paper-filtered, and concentrated 50-60-fold by negative pressure dialysis. Concentration was carried out at 4° using 0.39-in. dialysis casing (Union Carbide). Concentrate was dialyzed against normal saline, centrifuged at 100,000 *g* for 1 hr, and the pellet was discarded prior to estimation of the total protein content of the supernatant by the Lowry method. The supernatant material retained its antigenic activity for at least 8 months at −20°.

For control purposes, similar concentrated fluids were prepared from cultures of an established, H-2 unrelated, mouse cell line (L-929) and from H-2 identical C57Bl/6J normal adult fibroblasts (C57F) recently established in this laboratory. Also, similar fluids were prepared subsequent to repeated freezing and thawing of B-16 cells in serum-free RPMI-1640 at the same cell number to medium volume ratio as used for live monolayer-derived supernates. One-half of this killed cell suspension was processed immediately and fluid from the other half was collected and concentrated after incubation at 37° for 72-96 hr in order to allow the killed cells to undergo autolysis. The various supernatant preparations were coded as follows: (i) B-16SA, from live B-16 cell monolayers; (ii) B-16SA_k, from killed B-16 cells; (iii) B-16$SA_{k37°}$, from autolysed B-16 cells; (iv) C57FSA, from live monolayers of C57F cells; (v) L-929SA, from live monolayers of L-929 cells.

Preparation of antiserum. Antiserum to B-16SA was prepared in rabbits by repeated weekly, and later, biweekly, sc injections of approx 1 mg total protein of B-16SA. Initially, the B-16SA was injected as an emulsion in complete Freund's adjuvant. Booster injections were given in saline without adjuvant. Sera obtained 9 days after each sc booster injection or 4 days after an iv boost were found to contain maximal levels of specific antibody beginning at about 2 months after the initial sensitization.

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:*ipitation in agar*. All supernatant :ations were tested by double diffu- ı 1% Agar [Phosphate Buffered Sa- 'BS), pH 7.2] and by immunoelectro- sis (IEP) in 0.85% Agar (0.05 *M* bar- ›uffer, pH 8.2) against rabbit anti-B-

racterization of B-16 specific antigen. ıtinuous gel electrophoresis. Seven ıt polyacrylamide analytical gels were red according to the method of Davis [wo-hundred microliters of B-16SA ning 80 mg of sucrose and having a ›rotein content of 250 μg were layered p of a 3% stacking gel. A loading ıt of 2 mA/gel was applied for approx ı to complete the electrophoretic sep- n. Longitudinal sections of the gels ixed and stained for protein with Ani- lue-black or Buffalo black. Unfixed ıdinal sections were assayed for anti- ›cation by double diffusion in agar. arable gels were run with glycerol sub- d for sucrose and were stained for ›rotein with Alcian blue or for carbo- :es with Periodic Acid Schiff stain.

ıadex column chromatography. Three :ers of B-16SA containing 0.3 g of ›se was applied to a 2.5 × 45-cm col- ›f Sephadex G-150 and eluted at 4° BS pH 7.4. The effluent was collected ıl columns at a flow rate of 10 ml/hr :reened at 216 and 280 nm on a Gil- !40 spectrophotometer. Serial 15-ml were concentrated 10-fold by negative re dialysis and screened for B-16 spe- ıtigen by IEP and double diffusion.

ıunogenicity of B-16SA in C57Bl/6J C57Bl/6J male mice (18–20 g) ob- from the Jackson Labs (Bar Harbor,) were injected at weekly intervals c and ip with either (i) 50 μg B-16SA, ı μg C57FSA, (iii) 50 μg L-929SA, olyacrylamide isolated B-16 specific ın of B-16SA, or (v) whole frozen- B-16 cells (1 × 10^6). The antibody were determined by mixed hemad- ›n (3) 1 week after the last immuniz- iection (approx 2 months after initial on) using pooled serum.

ılts. Rabbit anti-B-16SA showed sev- recipitin lines in IEP with B-16SA 1A). Some components with similar ties were also present in the L-929SA and/or the C57FSA. Repeated (3×) absorption of the antiserum for a minimum of 2 hr each at 4°, RT and 37° with a saline-insoluble lyophilized powder of L-929, or a single absorption for 1 hr at 37° with L-929SA, removed all reactivity against L-929SA and removed reactivity against the most anodally migrating component of C57FSA and B-16SA (Fig. 1B and D). Absorption with C57 tissue (5×) powder or C57FSA (1×) removed all reactivity against both L-929SA and C57FSA. Two antigenically identical components with slightly different beta mobilities could still be detected in the B-16SA after extensive absorption of the anti-B-16SA serum with C57 tissue powder or C57FSA (Fig. 1C). The B-16 specific reactivity could be completely removed, however, by a single absorption with B-16SA or with a saline-insoluble lyophilized powder of B-16 tumor tissue derived from sc growth in C57Bl/6J mice.

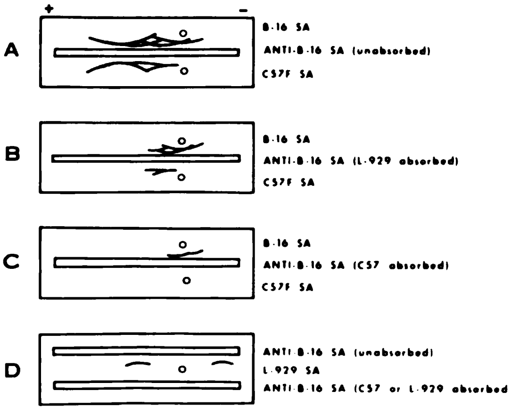
+
–
A
B-16 SA
ANTI-B-16 SA (unabsorbed)
C57F SA
B
B-16 SA
ANTI-B-16 SA (L-929 absorbed)
C57F SA
C
B-16 SA
ANTI-B-16 SA (C57 absorbed)
C57F SA
D
ANTI-B-16 SA (unabsorbed)
L-929 SA
ANTI-B-16 SA (C57 or L-929 absorbed)

FIG. 1. Immunoelectrophoresis patterns of B-16SA, C57FSA, and L-929SA run against unabsorbed and absorbed rabbit anti-B-16SA. The B-16 specific antigens (C) could no longer be detected after the antiserum was absorbed once with B-16 lyophilized powder or with B-16SA.

Double diffusion in agar (Fig. 2) with unabsorbed anti-B-16SA showed an antigenic component common to B-16SA, C57FSA, and L-929SA. A second B-16SA antigen was absent in L-929SA but displayed partial identity with a C57FSA component. A third antigen was detectable only in the B-16SA. The antigen common to all three supernates and the antigen limited to the B-16SA and C57FSA could no longer be detected when the antiserum was ab-

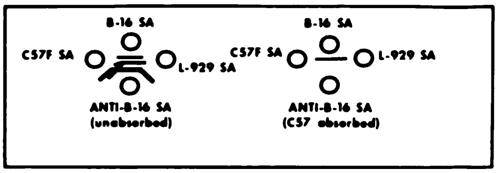

FIG. 2. Immunodiffusion patterns of B-16SA, C57FSA, and L-929SA against rabbit anti-B-16SA. The component which is common to all three supernates and the component which shows partial identity in the B-16 and C57 supernates are both removed by absorption with C57. The spur on the B-16SA is not detectable after absorption. However, the component which is present only in the B-16SA remains after C57 absorption and can only be removed by absorption with B-16.

sorbed with C57FSA or C57 powder. After extensive absorption with C57, a single line of reactivity remained against the B-16SA only. The B-16 specific reactivity could be removed only by absorption with B-16. An antigen identical to the B-16SA specific component could be detected in autolysed B-16 supernatant but not in non-autolysed killed cell supernatants (Fig. 3).

Rabbit anti-B-16SA also showed specific activity against B-16 cell membranes in the indirect immunofluorescence assay. Again, this activity could be removed by absorption with B-16SA or B-16 powder but not with similar preparations from either C57 or L-929 cells (Fig. 4).

Fourteen-sixteen protein staining components could be resolved in the B-16SA by discontinuous polyacrylamide gel electrophoresis. A line drawing representation of double diffusion in agar of one longitudinal section of B-16SA gel against anti-B-16SA is shown in Fig. 5. Two B-16 specific components were detected; the extreme anodal migration of the more prominent component contrasts with its slight anodal migration in IEP suggesting that the extreme migration in gel is a function of small size rather than large net negative charge. Carbohydrates were not demonstrable in areas of the gel in which B-16 specific antigen appeared.

The elution profile of the B-16SA on G-150 Sephadex is shown in Fig. 6. The antigen common to B-16SA, C57FSA, and L-929SA eluted in the first peak after the void volume. The location of the peak suggests a mol wt range of 70,000–150,000. The G-150 isolated B-16 specific antigens were reapplied to a calibrated G-75 column and their molecular sizes were determined. The major antigen appeared to have a mol wt of approx 25,000 and the minor antigen of a mol wt of 20,000.

Mice immunized with either unfractionated B-16SA or with the polyacrylamide gel derived fraction developed a serum antibody response to the B-16 cells, as measured by mixed hemadsorption, which was comparable to that developed by animals immunized with whole B-16 cells. Mice immunized with L-929SA or C57FSA did not develop and anti-B-16 antibody (Table I).

Discussion. At least one, and possibly two, specific membrane-associated antigens can be detected in the supernatants of cultured B-16 cells. The fact that these antigens remained in solution after centrifugation at 100,000 *g* suggests that they are not large membrane fragments. Furthermore,

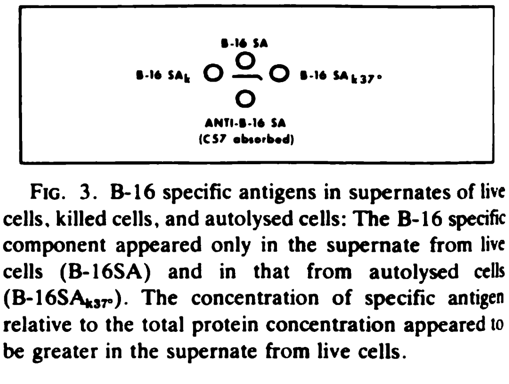

FIG. 3. B-16 specific antigens in supernates of live cells, killed cells, and autolysed cells: The B-16 specific component appeared only in the supernate from live cells (B-16SA) and in that from autolysed cells (B-16SA$_{k37°}$). The concentration of specific antigen relative to the total protein concentration appeared to be greater in the supernate from live cells.

TABLE I. ANTIBODY RESPONSE IN SOLUBLE ANTIGEN-SENSITIZED C57Bl/6J MICE.

Sensitizing antigen[b]	Antibody titer[a] vs B-16 cells	 vs L-929 cells
C57FSA	<1:10	<1:10
L-929SA	<1:10	1:640
B-16SA[c]	1:320	<1:10
Killed whole B-16 cells	1:512	ND[d]

[a] As measured by mixed hemadsorption using pooled serum.

[b] All antigen preparations were given both sc and ip in multiple doses over a period of 2 months.

[c] Polyacrylamide gel isolated B-16 specific antigen.

[d] Not done.

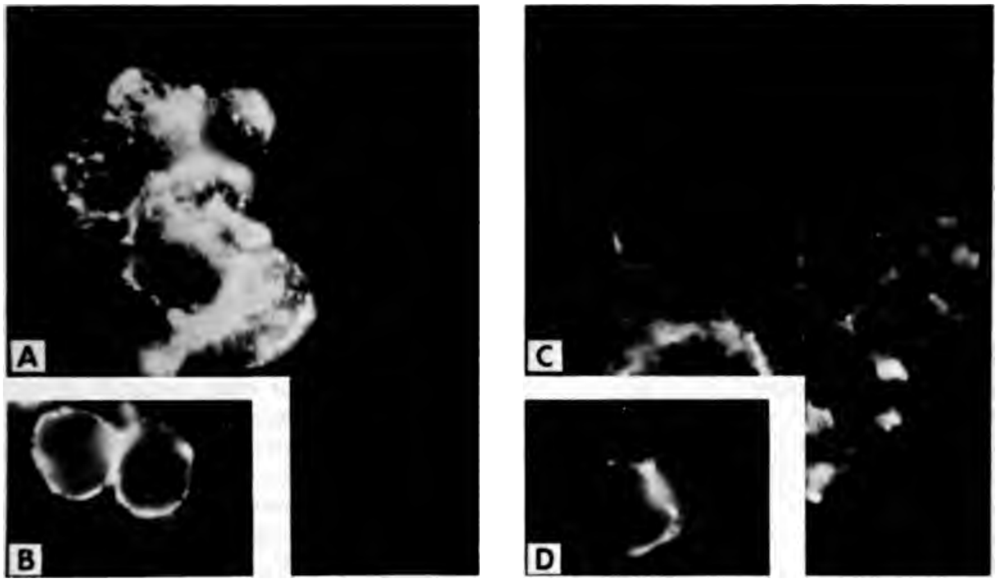

FIG. 4. Indirect immunofluorescence staining of B-16 cell membranes by rabbit anti-B-16SA and anti-rabbit gG-FITC: Anti-B-16SA was absorbed (A) with C57 lyophilized saline insoluble tissue powder (B) with C57FSA C) with B-16 lyophilized saline insoluble tissue powder (D) with B-16SA. Magnification ×400.

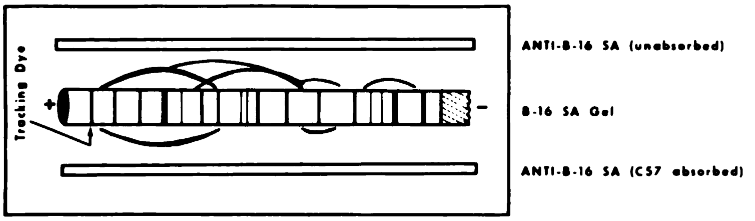

FIG. 5. Immunodiffusion pattern of B-16SA 7% polyacrylamide gel. The approximate positions of protein-taining bands in the gel were derived by staining one longitudinal section of the same gel which was tested in louble diffusion. There appear to be two components, migrating at 10–13 and 30–34 mm, respectively, from the racking dye which are B-16 specific. Absorption of the antiserum with B-16 removed this specific reactivity.

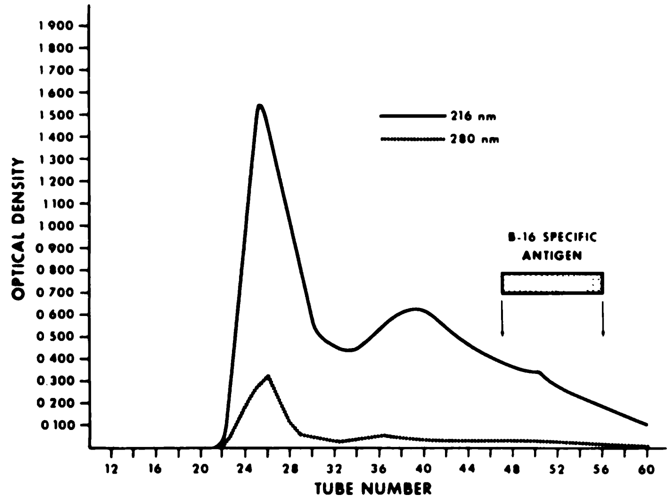

FIG. 6. Elution profile of B-16SA: The major B-16 specific antigen eluted in tubes 47–51 and the minor pecific antigen in tubes 47–56 on G-150 Sephadex. The antigen common to B-16SA and C57FSA eluted in tubes 7–46. The antigen which appeared in L-929SA as well as B-16SA and C57FSA eluted in tubes 32–46. No ntigenic activity was associated with the first peak (tubes 22–32).

the antigens were found to be soluble in distilled water and we believe that they may represent spontaneously shed, soluble, membrane-derived tumor-specific antigens. The B-16SA antigen which also appeared in L-929SA and C57FSA may be species-specific, and the antigen which was common to B-16SA and C57FSA but not present in L-929SA may be an H-2 antigen. This was not confirmed directly, however.

The fact that isolated B-16 supernatant antigens can induce an anti-B-16 cell membrane antibody in C57Bl/6J mice as well as in rabbits clearly demonstrates that the supernatant antigen is membrane associated. We have reported previously (3) that the serum and renal immune complex deposits from mice bearing a progressive B-16 tumor contain a material which is antigenically identical to the specific supernatant derived material and is smaller than 50,000 mol wt.

The relatively small size of the supernatant fluid derived B-16 specific antigen(s) in this study contrasts with the mol wt estimate of 150,000-200,000 for culture medium derived B-16-associated glycoprotein antigens reported by Bystryn *et al.* (6). Their material was not entirely B-16 specific, however, as it also appeared to some extent in the medium of C57 fibroblasts. This material may correspond to the larger, common antigens in our supernatant preparations. Furthermore, we were unable to detect carbohydrate associated with the B-16 specific antigen in PAS and Alcian blue-stained polyacrylamide gels. The relatively low sensitivity of these stains does not allow us to say that our material was definitely not a glycoprotein, however.

In vivo release of a human melanoma antigen into tumor cyst fluid and urine has been reported (8). The material was found to be mitogenic for lymphocytes from several melanoma patients but not for normal human lymphocytes. The soluble human melanoma antigen was similar to the supernatant-derived B-16 specific antigen in that it showed a beta mobility in IEP and its presence in urine indicated a mol wt of under 40,000.

We believe that the release of B-16 specific antigen may occur as a normal part of cell membrane turnover during which other, nontumor specific membrane antigens are also released. The release of soluble tumor antigen *in vitro* by living cells and by autolysis of dead cells may be paralleled *in vivo*; such *in vivo* release may further be related to the failure of demonstrably sensitized hosts to reject their tumors (3).

Summary. Serum-free supernatant fluids from monolayer cultures of B-16 mouse melanoma cells were found to contain a soluble membrane associated tumor-specific antigen. The 100,000 *g* supernatant of the culture fluid induced an antibody response to the B-16 cells both in rabbits and in the mouse strain of origin (C57Bl/6J).

Similar supernatant fluids derived from an unrelated cell line (L-929) or from normal C57Bl/6 fibroblasts did not contain the B-16 specific material. Preliminary results indicate that the B-16 specific material is a protein of low molecular weight which is released into the culture fluid chiefly by living cells and, to a lesser extent, by autolysing cells.

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ctuations of Unbound Whole Blood Polyamine Levels during the Menstrual Cycle (39333)

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polyamines, putrescine, spermidine and spermine (Spm), appear to be tous in animal tissues. The only route of polyamine biosynthesis in ials involves the decarboxylation of ine to form putrescine, which reacts he *N*-propylamine moiety of decar- ted *S*-adenosylmethionine to form idine, and in a similar manner spermi- acts with decarboxylated *S*-adenosyl- nine to form spermine. Although the logical role of these cations remains eluciated, they have been implicated ing an important role during growth llular transformation. Their level in- s dramatically along with their respec- iosynthetic enzymes at the onset of s growth processes (1). Several hor- , including luteinizing hormone (2), iol (3, 4), and testosterone (5) di- affect polyamine biosynthesis; fur- ore, studies (6, 7) indicate that one or of the polyamines, at physiological itrations, can substitute for hormones liating specific biologic responses.

ent clinical reports (8, 9) have dem- ted that various neoplastic states are panied by increased levels of tissue irculating polyamines; and, in some successful therapy leads to a decrease concentration of these compounds nother possible medical application of nine determinations relates to identi- n of cystic fibrosis homo- and hetero- s (10, 11). In particular, our labora- bserved that male, but not female, and heterozygotes for cystic fibrosis the most common lethal inherited dis- ffecting Caucasians (12), were charac- l by a significantly higher Spd/Spm in unhydrolyzed whole blood when red to control subjects (11). The ob- on that male, but not female, CF patients displayed this abnormality is not consistent with the accepted mode of inheritance of this genetic disease: i.e., autosomal recessive inheritance (12). Close inspection of the polyamine levels and Spd/Spm ratios from female controls and female homo- and heterozygotes for CF suggested that their ratios are subject to greater amounts of variability than male values. This observation suggested that a sex-related hormone(s) may be influencing the female Spd/Spm ratio during the menstrual cycle. For this reason, we have examined unhydrolyzed whole blood Spd and Spm concentrations and Spd/Spm ratios in four normal females, three normal males, and one ovariectomized female, over a period of 4 weeks, to encompass the menstrual cycle.

Materials and methods. Subjects. Blood samples were obtained from normal volunteers who were housed at the National Institutes of Health for the duration of these studies. A routine medical examination showed all to be in good health. The ages of the normal males were 19, 19, and 20. The normal females, ages 19, 19, 19, and 20, had regular menstrual cycles of 23, 27, 27, and 31 days. One of the four was placed on an oral contraceptive containing 1 mg of ethynodiol diacetate and 50 μg of ethinyl estradiol (Demulen, Searle & Co., Chicago) 30 days after the initial studies, and her Spd and Spm concentrations were determined for an additional 22 days.

The ovariectomized female available for these studies was 25-yr old, in good health, and not taking hormonal supplements. Sixteen months previously, she had been found to have a differentiated adenocarcinoma of the endometrium and shortly thereafter underwent total abdominal hysterectomy, bilateral salpingo-oophorectomy, and appendectomy.

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eserved.

Polyamine extraction. Whole blood was obtained in a fasting state from all the individuals and extracted within 30 min of collection. Extraction of whole blood polyamines was carried out as follows, using plastic containers when possible. Whole blood (10 ml) was drawn into a syringe containing 1.0 ml of 3.0% sodium citrate, mixed, added to 10 ml of 3.0% $HClO_4$, allowed to stand at 5° for 20 min and centrifuged at 4500 *g* for 20 min. The supernatant was decanted and stored, and the pellet re-extracted as above. The supernatants were pooled, then neutralized with 4 *N* KOH and stored at 4° for 16 hr. After decanting, 2.0 ml of 3.0% sulfosalicylic acid was added (to assure precipitation of any residual protein), the resulting precipitate removed by 10,000 *g* centrifugation, and the extract concentrated to dryness by lyophilization. Samples were then resuspended in 2.0 ml of sodium citrate buffer (pH 2.2, 0.2 *M*) and centrifuged at 4500 *g* for 10 min to remove residual $KClO_4$. Previous studies using [^{14}C]Spd and [^{14}C]Spm, added to whole blood and carried through the extraction procedure, had shown that recoveries averaged 66% and varied by less than 5% for either polyamine.

It should be noted that "polyamines" in this study refer to free, as opposed to total, blood polyamines which would also include those known to be covalently bound. Determination of total blood polyamines requires acid hydrolysis with 6 *N* HCl at 110 *c* or a similar procedure and is the technique most often utilized when studying polyamine concentrations in fluids from cancer patients (1). We have elected not to hydrolyze in view of the following: (i) the original observation that polyamine levels are abnormal in the blood of cystic fibrosis patients was made on unhydrolyzed blood (10); (ii) our subsequent conformation of the original observation was made on unhydrolyzed blood (11); and (iii) the initial suggestion that blood polyamine levels may fluctuate in females was obatined on unhydrolyzed blood.

Polyamine separation and quantification. Polyamines were separated and quantitated using a KCl-potassium citrate H_2O buffer, as previously described (13). Of the above supernatant, 0.5 ml (equivalent to 2.5 ml whole blood) was applied to an amino acid analyzer column (0.9 × 23 cm), containing PA-35 resin (Beckman Instrument Co., Palo Alto, Calif.). Replicate samples routinely showed greater than 95% agreement between runs.

Hormone determination. Plasma samples collected at the same time as whole blood samples for polyamine determinations were analyzed for estradiol and progesterone by Bio-Science Laboratories (Van Nuys, Calif.).

Statistics. Female Spd/Spm ratios appeared to be cyclic in nature, suggesting that it may be unreasonable to assume that an individual female ratio belongs to a known frequency distribution, thereby precluding the use of a parametric test for statistical comparison of male to female ratios. To compare female to male ratio variance we have used the Wilcoxon–Mann–Whitney *U* test, a nonparametric test.

Results. Male ratios and concentrations. Male Spd/Spm ratios and concentrations are shown in Fig. 1A and B, respectively. The

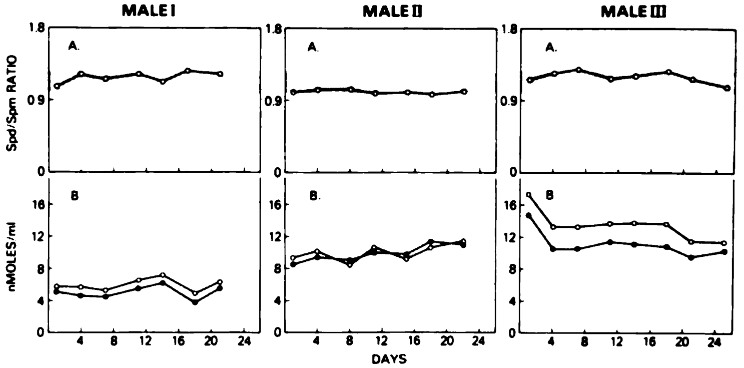


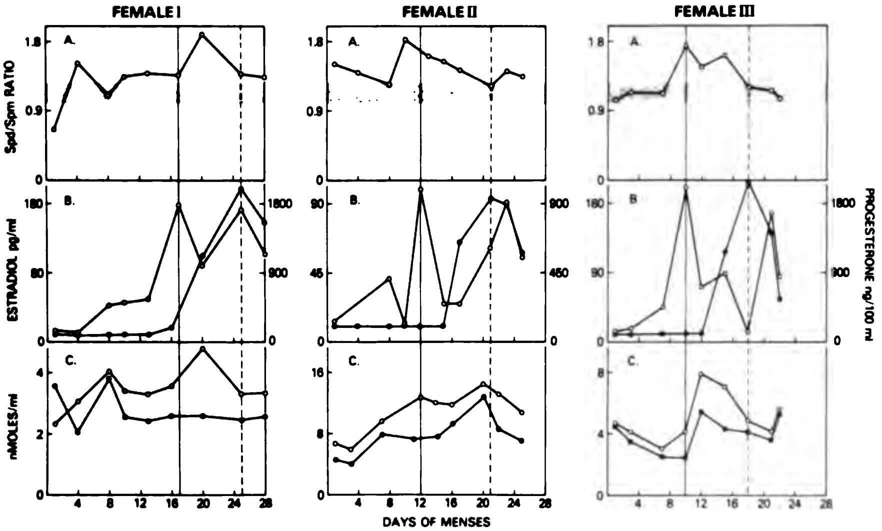
FIG. 1. (A) Male Spd/Spm ratio profiles. The shaded areas indicate the cumulated 1 SD interval obtained for Males I, II, and III. (B) Male Spd (-O-) and Spm (-●-) concentrations.

ın and 1 SD for the cumulated ($n = 22$) e ratios is 1.14 ± 0.11. Of the 22 indiıal male ratios, all but one fell within the) interval. Although showing more flucion than their respective ratios, the ac Spd and Spm concentrations (Fig. 1B) ear to be stable for a given individual. vever, unlike the individual ratios, ch varied little from one male to the next are similar to our previous results (11), individual Spd and Spm concentrations ed from one male to the next (Fig. 1B). example, the mean ± SE for Spd contrations of Male I and Male III were 5 ± 0.25 and 13.69 ± 0.67 nmole/ml, ectively.

ormal female ratios and concentrations. ontrast to the male Spd/Spm ratios, the · normal females showed substantial tuation in their ratios (Figs. 2A and 3A) when compared to male values were ıd to be significantly different ($P <$ 29). The mean cumulative ($n = 36$) fee ratio is 1.34 ± 0.25. Eighteen (64%) he female ratios fell above the 1 SD rval for males (shaded areas, Figs. 3A 4A) and 11 (39%) were greater than 3 However, only two ratios fell below the 1 SD interval.

gure 2B provides the estradiol and proerone levels determined for three of the normal females. The highest values obtained for the midcycle peak of estrogen and the progesterone surge are indicated by vertical solid lines and hatched lines, respectively (Fig. 2B). Female ratios appear to rise at some point after the last day of menses and fall before the onset of the next menstrual cycle. As was typical of the male Spd and Spm concentrations (Fig. 1B), the female Spd and Spm concentrations (Fig. 2C) varied from one individual to the next. However, unlike the individual male concentrations, which appear to be stable over the period of the study, individual females, when compared to males, show a high degree of fluctuation from one sampling to the next.

Female receiving oral contraceptives. The Spd/Spm ratios of a fourth normal woman were determined for a period of 32 days, after which she was placed on the oral contraceptive Demulen and the Spd/Spm ratios determined for an additional 22 days. Ratios before receiving the oral contraceptive (Fig. 3A) had the same appearance as the other three women's (Fig. 2A) and were characteristic of an increase well above the male 1 SD (shaded area Fig. 3A). As shown, this increase occurred approx 15 days after the first day of menses and fell substantially at about Day 20. After receiv-



G. 2. (A) Female Spd/Spm ratio patterns with male cumulated 1 SD interval indicated (shaded areas). Day 1 s the first day of menses. (B) Plasma estradiol (-O-) and progesterone (-●-) levels. The highest values ned for the first estradiol peak and the highest value obtained for progesterone during the menstrual cycle are ıted by the vertical solid and hatched lines, respectively. (C) Female Spd (-O-) and Spm (-●-) concentrations.

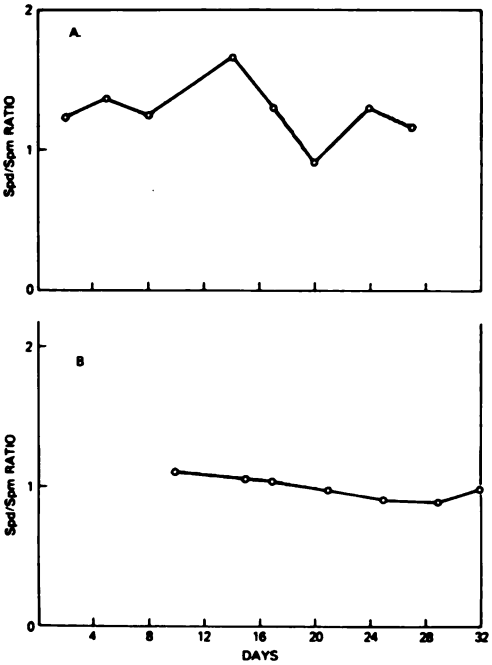


FIG. 3. (A) Female Spd/Spm ratio profile before receiving oral contraceptive. Day 1 equals first day of menses. The shaded area indicates the cumulated male 1 SD interval. (B) Female Spd/Spm ratio profile after receiving oral contraceptive (shaded area as in A).

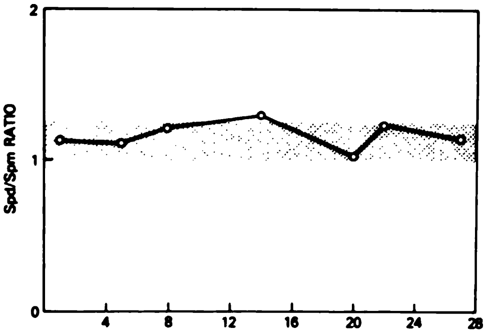


FIG. 4. Ovariectomized female Spd/Spm ratio pattern. The shaded area indicates the cumulated male 1 SD interval.

ing the oral contraceptive, however, her ratio pattern showed very little fluctuation: the characteristic finding obtained with males (Fig. 1A). The mean Spd/Spm ratio, as well as the SEM (a reflection of ratio variance), while on hormonal supplements, was significantly ($P < 0.02$) lower than the mean figure ± SEM obtained in the first study (0.99 ± 0.08 vs 1.25 ± 0.19, respectively).

Ovariectomized female ratios. The Spd/Spm ratios obtained for an ovariectomized woman are provided in Fig. 4. Unlike the noraml women, the ovariectomized woman showed very little fluctuation and provided a Spd/Spm ratio pattern similar to the males'. Only one of the Spd/Spm ratios obtained for the ovariectomized woman fell outside the 1 SD interval for men (shaded area, Fig. 4) and it came close to being within this interval.

Discussion. These studies demonstrate that over extended periods of time, blood samples from normal women exhibit a higher Spd/Spm ratio variance than to specimens from men. The increased variance appears to be a reflection of the variation during the menstrual cycle, suggesting that a sex-related hormone(s) influences whole blood Spd and Spm concentrations.

This is also supported by the observations that: (i) a woman who possessed a Spd/Spm ratio pattern characteristic of three other women assumed a male ratio profile after taking an oral contraceptive containing a progesterone analog as the active ingredient, and (ii) an ovariectomized woman exhibited a Spd/Spm ratio profile very similar to men's.

Our findings suggesting an interrelationship between hormones and polyamines are in keeping with previous studies: (i) administration of 17-β-estradiol leads to increased putrescine, spermidine, and spermine, as well as their respective biosynthetic enzymes in castrated rat uterus (3); (ii) castrated rats are characterized by a marked reduction in polyamine concentrations as well as biosynthetic enzymes in ventral prostate, all of which increase after injection of testosterone propionate (5); (iii) in rat ovary, luteinizing hormone and human chorionic gonadotropin stimulate the first and rate-limiting enzyme in polyamine biosynthesis, ornithine decarboxylase (2); (iv) recent data suggest that ornithine decarboxylase may, in part, be regulated by cyclic AMP (14); (v) spermidine, at physiological concentrations, can substitute for glucocorticoid in promoting milk production in cultured mammary cells (6); and (vi) spermine and spermidine can mimic the action of insulin toward lipid and glucose metabolism in cultured adipose tissue cells (7).

It has been shown that polyamine concenns are elevated in the blood and urine ividuals with a variety of different neo- (8, 9) and that the polyamine levels ıe upon remission (9). These observa- have prompted speculation concerning tilization of polyamine measurements index of malignant disease (9, 15). In ion to abnormal levels in cancer, other sed states such as cystic fibrosis (11) olycythemia vera (16) appear to be iated with aberrant blood unbound mine concentrations and/or Spd/Spm . An inherent requirement of utilizing cific metabolite as a diagnostic tool, is the normal population be clearly de- . Our data suggest that major differ- exist with respect to whole blood Spd/ ratios and concentrations in men, as ared to women. Since female values ate to a greater extent, it appears nec- to measure blood polyamines in n at a specific time in the menstrual if valid data for clinical diagnoses are obtained. Men, on the other hand, nearly constant Spd/Spm ratios over ded periods of time, suggesting that mal polyamines in whole blood may eful in the diagnosis, or in following inical course, of some maladies such as r and cystic fibrosis.

ally, in view of the fact (1) that polya- exist in covalently bound as well as orms, it will be of interest to determine magnitude of polyamine concentra- and spermidine/spermine ratio fluctua- observed in females during these stud- unhydrolyzed samples are even more unced after hydrolyzing samples. If appears evident that the time samples btained from female cancer patients ontrol subjects for polyamine analysis be coordinated with specific times in enstrual cycle.

ımary. Perchloric acid-extractable blood spermidine and spermine con- ıtions were determined over a 4-week l in three men, four women, and one ctomized woman. Individual male idine/spermine ratios showed little ation and similar values were obtained ch of the three males studied. Male idine and spermine concentrations, al- ı stable for each male, varied from ıbject to the next. Individual female spermidine/spermine ratios, as well as individual concentrations, fluctuated substantially when compared to those values obtained for males; female ratios appear to rise and fall as a function of the menstrual cycle. The spermidine/spermine ratios obtained from a normal female receiving oral contraceptive, as well as those from an ovariectomized female, were characteristic of values obtained from men. It is suggested that a sex-related hormone(s) influences both the spermidine/spermine ratio and spermidine and spermine concentrations in females.

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Steroid Synthesis by Ovarian Follicles in Response to LH and $PGF_2\alpha$ (39334)

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Shemesh and Hansel (1) reported that changes in the concentration of testosterone in bovine jugular vein plasma are quite similar to those that occur in plasma estrogen concentrations during the estrous cycle. These changes include a marked rise in plasma testosterone concentration during proestrus immediately following the decline in plasma progesterone levels. Evidence that the mammalian ovary secretes androgens was first provided by Hill (2). Hilliard *et al.* (3) reported that rabbit ovaries can produce androgens *in vivo*, as has been shown *in vitro* (4). The secretion of androgen, estradiol, and progesterone into the blood of the ovarian vein of sheep is significantly increased by injection of gonadotropins; the steroid levels begin to rise within 30 min of injection (5). It was also found by Shemesh and Hansel (6), that arachidonic acid injected into the bovine corpus luteum *in vivo* was converted into prostaglandin F and that the large amounts of prostaglandin F produced in this way resulted in elevated plasma estrogen concentrations.

The present investigation was undertaken to measure testosterone production by the isolated bovine follicular tissue and to determine the effects of exogenous LH and $PGF_2\alpha$ on this production. Estrogen and progesterone production were also examined to ascertain whether changes in androgen biosynthesis are accompanied by changes in synthesis of these steroids.

Materials and methods. Animals and tissues. Ovaries were removed from four normal Holstein heifers at the 16th or 17th day of their estrous cycles through flank laparotomies made under local anesthesia. All follicles, ranging in size from 0.4 to 1 cm in diameter, were dissected free, minced, pooled between ovaries, and incubated in Krebs-Ringer bicarbonate buffer at 37° for 2 hr as described by Hansel (7). Most of the follicles minced were small (approx 0.4-0.6 cm in diameter). However, ovaries from each animal contained one or more large (approx 1.0 cm) follicles. Five incubation vessels were used for each treatment for each animal. Samples were frozen on Dry Ice at the end of each incubation. Each vessel contained 50 mg of follicular tissue and the stimulatory levels of LH (LH-B13, 5 μg/ml) or $PGF_2\alpha$ Tham salt (5 μg/ml). Previous work has shown that these levels of LH and $PGF_2\alpha$ will cause maximum stimulation of progesterone biosynthesis in bovine luteal tissue slices (8).

Extractions. The samples were thawed, homogenized, and methanol was added to a concentration of 60%. Approximately 5000 cpm each of tritiated estradiol, estrone, progesterone, and testosterone (80-110 Ci/mmole; New England Nuclear) were added to each vessel. The homogenate was then transferred to stainless steel centrifuge tubes with repeated washings of 60% ethanol. The homogenate was shaken for 3 hr and stored overnight at −18°. The tubes were then spun in a refrigerated centrifuge at approx 20,000 *g* for 45 min to remove precipitated protein and lipid. The supernatant was collected and taken to dryness on a rotary evaporator. The residue was dissolved in 1 ml of ethanol and transferred with repeated 50-ml washings of benzene and light petroleum (1:1) to a separatory funnel. The benzene:light petroleum solution was extracted twice with 25 ml of 0.4 NaOH to yield a phenolic fraction. The combined alkali extracts were acidified with HCl and extracted with ether (3 × 25 ml). The ether was washed with water (2 × 1/20th vol) to neutrality. After removal of the organic solvents the residues were each transferred with 4 ml of ethanol into small test tubes and dried under N_2 at 45°. The remaining benzene:light petroleum solution was washed with water and the organic phase was dried in a rotary evaporator. The resi-

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vas transferred to a test tube with ol and dried under N_2 at 45°.

'omatography. Column chromatogra- ıf the estrogens was accomplished on ıdex-LH 20 with benzene:methanol 0). The neutral steroids, progesterone, ;stosterone were also separated by col- chromatography on Sephadex-LH 20 hexane:benzene:methanol (90:10: as described by Carr *et al.* (9). The ge recoveries of known amounts added replicates were (mean ± SE) 91 ± 8% ıtrone, 72 ± 13% for estradiol, 87 ± or progesterone, and 76 ± 9% for ıterone.

lioimmunoassays. The method of rnkamp and Hansel (10) was em- d for the estrogen assays. The method ıbed by Hixon *et al.* (11) was employed ırogesterone radioimmunoassay and f Shemesh and Hansel (1) was used for terone radioimmunoassay.

'istics. The data were subjected to sis of variance and least significant dif- ;es were determined by Tukey's hsd ɔd (12).

ults and discussion. the effects of add- H or $PGF_2\alpha$ to the incubation media ıthesis of testosterone by bovine follic- issue are shown in Fig. 1. Incubation failed to cause an increase in testoster- ($P > 0.05$). Additions of LH and α each caused increased testosterone ;sis ($P < 0.01$ and $P < 0.01$, respec-). Estradiol synthesis (Fig. 1) was also lated by addition of LH ($P < 0.01$), 'nthesis due to addition of $PGF_2\alpha$ was ıatistically significant ($P > 0.05$). Es- synthesis was increased from 6 to 9 ıy addition of LH and from 6 to 10 ng/ 'G$F_2\alpha$, but neither increase was signifi- $P > 0.05$). The effects of LH and PGF *vitro* synthesis of progesterone by bo- ollicular tissues are also shown in Fig. ıere was no increase in progesterone ;sis due to incubation alone, addition [, or addition of $PGF_2\alpha$ ($P > 0.05$).

;se results show that bovine follicles at 5th or 17th day of the estrous cycle ın appreciable testosterone (12.1 ng/ tradiol (4.4 ng/g), and progesterone ıg/g). Both LH and $PGF_2\alpha$ stimulated the synthesis of relatively large amounts of testosterone and estradiol synthesis was stimulated by LH. Increased plasma estradiol levels noted in previous experiments following intrauterine administration of $PGF_2\alpha$ (11) and intraovarian administration of arachidonic acid (6), suggest that $PGF_2\alpha$ stimulates estrogen production by the follicle, even though the stimulated estradiol synthesis in this experiment was not statistically significant. The failure of LH or $PGF_2\alpha$ to stimulate *in vitro* progesterone synthesis may have reflected rapid conversion of progesterone into testosterone.

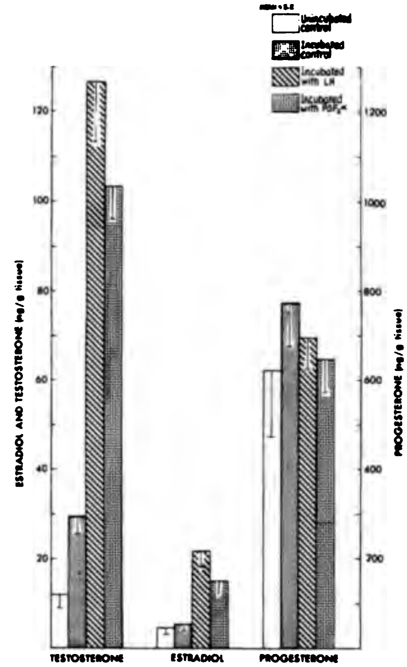

FIG. 1. Mean testosterone, estradiol 17β, and progesterone biosynthesis by bovine follicular tissue incubated alone and with added $PGF_2\alpha$ and LH. Vertical lines represent standard errors of the means.

The high level of testosterone found and its stimulation by either LH or $PGF_2\alpha$ are in keeping with the high peripheral plasma testosterone levels found in the cow before the onset of estrus (1). Lindsay and Robinson (13) and Shemesh *et al.* (14) suggested the possible estrus-inducing activity of testosterone and other androgens. However, it is not clear whether testosterone per se, plays a role in the complete manifestation of estrus, or whether it serves only as a precursor for estrogen. Recent evidence obtained for androgen-insensitive mice suggests that androgens may be needed for normal ovarian function (15). Rao (16) reported that testosterone and dihydrotestosterone enhanced [^{3}H]PGE_1 binding to bovine corpus luteum cell membranes, but estradiol-17β and estrone inhibited binding.

The precursors of the testosterone synthesized in response to LH and $PGF_2\alpha$ remain questionable. It is possible that the precursor is progesterone, since progesterone values did not increase concomitantly with the testosterone increase. On the other hand, one would expect a significant rise in progesterone biosynthesis, as demonstrated by Stoklosowa and Nalbandov (17) and others in rat follicular tissue cultures. It is possible that the Δ^4 pathway for testosterone synthesis through progesterone was activated, but one cannot rule out the possibility that the Δ^5 pathway for testosterone synthesis exists in the bovine follicle. Existence of the Δ^5 pathway could also explain the lack of progesterone stimulation by LH and $PGF_2\alpha$. YoungLai (18) suggested that Δ^5 androstene-3β, 17-diol may be a logical precursor in rabbit follicles. Stoklosowa and Nalbandov (17) reported that 10 ng of LH was able to cause maximal progesterone synthesis in rat follicular tissue cultures, while 10 times that amount of LH was required for maximal estrogen synthesis. The higher level did not increase the rate of progesterone synthesis. Thus, the high level of LH we used in the present study might explain the data obtained.

LH can terminate estrogen secretion and promote progestin secretion in the estrous rat (19). Thus, the high levels of LH used could have inhibited the aromatizing system. In the rat, the aromatizing system is located in the granulosa cells and is specifically stimulated by FSH (20). Moor *et al.* (21) indicated that whole sheep ovarian follicles less than 4.5 mm in diameter secreted considerable estrogen during the first 5 days of culture, but produced only small quantities of progesterone. Most follicles explanted at estrus produced only small amounts of estrogen *in vitro*, but synthetized large amounts of progesterone. *In vitro* studies indicate that follicles may contribute to preovulatory progestin secretion in the rat (17), and *in vivo* studies suggest that the same is true for the rabbit (22) and hamster (23).

Summary. Ovarian follicles isolated from bovine ovaries at the 16th and 17th days of the estrous cycle were minced, pooled, and incubated in Krebs–Ringer bicarbonate buffer at 37° for 2 hr. Samples were analyzed for testosterone, estrogens, and progesterone by radioimmunoassays. Incubation alone failed to cause an increase in testosterone (12.1 vs 29.3 ng/g of tissue; $P > 0.05$). Additions of 5 μg/ml of bovine LH or prostaglandin $F_2\alpha$ caused increases in testosterone synthesis to 125.0 ($P < 0.01$) and 103 ng/g ($P < 0.01$). Estradiol synthesis was also stimulated by LH ($P < 0.01$). However, stimulation due to addition of $PGF_2\alpha$ (5.1 to 15 ng/g) was not significant ($P > 0.05$). $PGF_2\alpha$ also caused a slight but nonsignificant increase in estrone secretion. Incubation failed to increase the progesterone concentration (623 vs 774 ng/g of tissue; $P > 0.05$). There was no increase in progesterone synthesis due to addition of either LH or $PGF_2\alpha$ ($P > 0.05$). The results show that bovine follicles produce much more testosterone than estrogen and that both LH and $PGF_2\alpha$ significantly stimulate testosterone synthesis in bovine follicular tissue without increasing net progesterone biosynthesis.

This study was supported, in part by USPHS Grant No. 69-2209. Thanks are due to Dr. G. E. Abraham and Dr. B. V. Caldwell for steroid antisera. The skilled technical assistance of Mr. R. Saatman and D. Gengenbach is gratefully acknowledged.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 90-94 (1976)

Inhibitory Effect of Large Doses of Propylthiouracil and Methimazole on an Increase of Thyroid Radioiodine Release in Response to Thyrotropin (39335)

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Chronic administration of antithyroid drugs lowers circulating thyroid hormone by blocking synthesis of thyroid hormones and thus produces goiter by augmenting thyrotropin (TSH)[1] secretion from the pituitary (1, 2). Although a single injection of antithyroid drug can block hormone synthesis within a few minutes, it is not known how soon pituitary release of TSH increases in response to antithyroid drugs. Thyroidal radioiodine release can be used as a sensitive measure of changes in TSH secretion (3). It is accelerated shortly after TSH (3) or small doses of propylthiouracil (PTU). Since blood TSH increased 24 hr after a single injection of PTU, and since blood PBI was normal during this period, Escobar *et al.* (4) hypothesized that PTU augmented TSH secretion by inhibiting deiodination of thyroxine (T_4) and reducing negative feedback, an argument requiring the assumption that deiodination of T_4 is an obligatory step in its action.

While we were studying some aspects of this hypothesis in rats, we incidentally found that large doses of PTU and methimazole (MMI) failed to augment thyroidal radioiodine release for a period of 29 to 34 hr. This is unexpected finding, since thyroidal radioiodine release is accelerated when recirculation of iodide is blocked, and since large doses of goitrogens are required to inhibit recirculation of iodide to monoiodotyrosine (1). A number of studies were thus performed to shed some light on this unexpected finding.

Materials and methods. One-hundred-thirty-six male Wistar rats, weighing 150 to 200 g, and 72 male DDY mice, weighing 20 to 30 g, were used in this experiment. The animals were fed a Remington low iodine diet (LID) or a moderately low iodine diet[2] (MLID) beginning 7 to 14 days before radioiodine injection. Beginning 28 to 48 hr after the injection of 20 μCi of ^{131}I, thyroidal radioiodine release was measured, with or without injection of anti-thyroid drugs (5). In Experiment 3, blood was obtained by cardiac puncture to measure plasma TSH, $^{131}I^-$ and PB^{131} I during measurement of thyroidal radioiodine release. Plasma TSH was measured according to the McKenzie (3) method, and plasma $^{131}I^-$ and PB^{131}I were measured by the trichloroacetic acid precipitation technique reported previously (6). In Experiment 5, the McKenzie method was also used to assess thyroidal response to exogenous TSH. The data were analyzed for statistical significance by the Student's *t* test. The *P* value less than 0.05 was considered statistically significant.

Results. Experiment 1. Effects of graded doses of methimazole (MMI) on thyroidal radioiodine release in rats fed a low iodine diet. For the first 60 hr, three groups of eight animals each received saline (0.5 ml) ip once daily. The half-life of thyroidal radioactivity during this period ranged from 51 to 56 hr, without a significant difference among the groups (Fig. 1). Seven to nine hours after the administration of MMI (7 or 15 mg), an increase of thyroidal radioiodine release was found in all animals. However, when 40 mg of MMI was administered, a latency of 29 hr was found before any increase of thyroidal radioiodine release could be detected. After the increase of radioiodine release, no difference in half-life of thyroidal radioactivity was found among the three groups.

Experiment 2. Effects of graded doses of propylthiouracil (PTU) on thyroidal radioio-

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[1] TSH, Armour Thytropar.

[2] Composition of this diet was shown in Endocrinology 79, 138 (1966).

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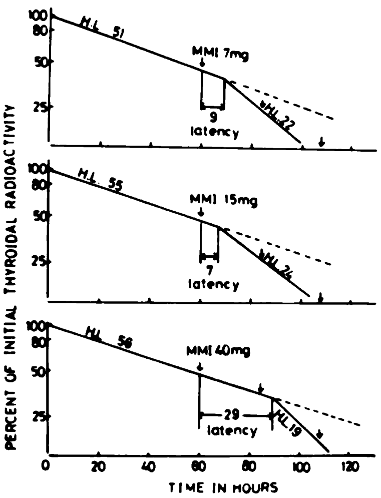

FIG. 1. Typical curve of thyroidal radioiodine release before and after methimazole. In this and subsequent figures: H.L., half-life of thyroidal radioactivity; latency, hours elapsed before an increase of thyroid secretion was detected; MMI, methimazole.

dine release in rats fed a low iodine diet. For the first 60 hr, four groups of eight animals each received gelatin solution (2%) sc daily. There was no significant difference in the thyroidal release among them (Fig. 2). When 1 or 5 mg of PTU was administered, a marked increase of thyroidal radioiodine release occurred immediately after the PTU. However, only a smaller increase of secretion occurred with 30 mg of PTU, which was followed by a final increase 30 hr later. The half-life of thyroidal radioactivity during intermediate and final increase was 31 and 15 hr, respectively. When 60 mg of PTU was administered, 34 hr elapsed before a significant increase was detected. There was no significant difference in the final release rate among the four groups.

Experiment 3. Effects of graded doses of propylthiouracil on thyroidal radioiodine release, plasma $^{131}I^-$, $PB^{131}I$, and TSH in rats fed a moderately low iodine diet. In the gelatin-injected controls, the half-life of thyroidal radioactivity was 104 ± 21 hr (mean ± SE) (Fig. 3). The administration of 5 mg of PTU produced a slight increase of thyroidal radioiodine release with a half-life of 67 ± 9 hr, which was followed by a final increase 12 hr later. When 60 mg of PTU was administered, a 34-hr latency was found before any increase of thyroidal radioiodine release occurred. The half-life of thyroidal radioactivity was slightly longer in the 5-mg PTU group than in the 60-mg PTU group.

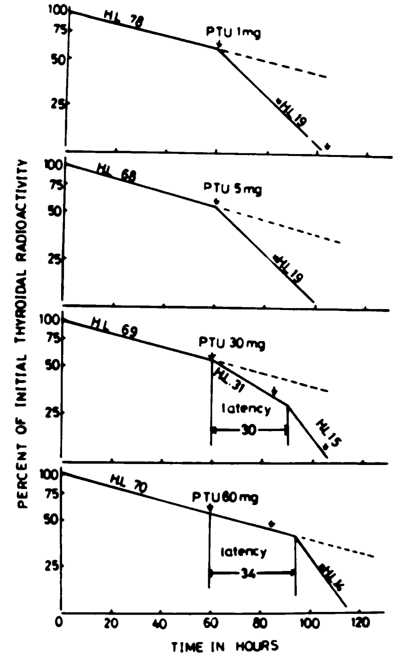

FIG. 2. Typical curve of thyroidal radioiodine release before and after propylthiouracil. PTU, propylthiouracil.

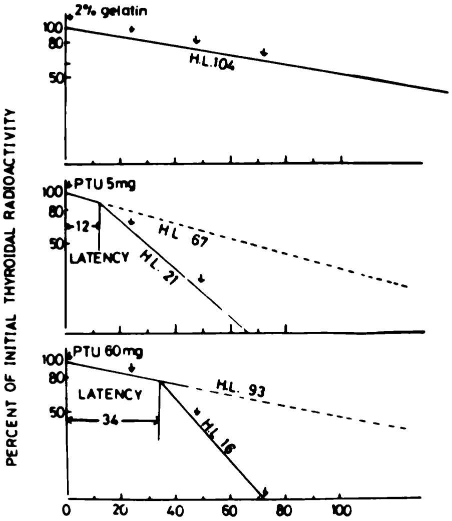

FIG. 3. Typical curve of thyroidal radioiodine release before and after propylthiouracil. Arrow indicates injection of gelatin or PTU. PTU, propylthiouracil.

Blood was obtained by cardiac puncture for plasma iodine analysis and TSH bioassay, just before and 24 as well as 52 hr after the initial dose of PTU. As shown in Fig. 4, plasma $PB^{131}I$ and $^{131}I^-$ were fairly constant throughout the experimental period in the gelatin-injected controls. PTU administration produced a marked increase of plasma $^{131}I^-$ and a slight decrease of $PB^{131}I$. As compared with the 5-mg PTU group, an increase of plasma $^{131}I^-$ in the 60-mg PTU group was less at 24 hr ($P < 0.05$).

Finally, plasma TSH activity was measured in 42 mice fed MLID for 7 days (Fig. 5). As compared with the control plasma, a marked increase of plasma radioactivity was found 24 and 52 hr after administration of 5 mg of PTU ($P < 0.001$ at 24 hr). An increase of plasma radioactivity was also found 24 hr after administration of 60 mg of PTU (control vs PTU 60-mg, $P < 0.01$), although the magnitude of the response was slightly less in the 60-mg PTU group than in the 5-mg PTU group.

Experiment 4. Effects of propylthiouracil and methimazole on thyroidal response to TSH in rats treated with thyroxine. In the first experiment, 27 animals were divided into three equal groups (Control, PTU, and MMI groups) and were fed MLID for 10 days before the experiment. Beginning 48 hr after ^{131}I injection, thyroidal radioiodine release was measured while the animals were receiving 15 μg of T_4 once daily ip. Thyroidal radioiodine release was exponential but slow because of depression of TSH. Administration of PTU (5 mg) or MMI (5 mg) once daily for 2 days had no consequence on thyroidal radioiodine release. With the last dose of goitrogens, 1 U of TSH was administered sc. Just before and 20 hr after the injection of TSH, blood was obtained for plasma $PB^{131}I$ analysis. As shown in Fig. 6 (left), the increase of plasma $PB^{131}I$ was less in the PTU group than in gelatin-injected controls ($P < 0.02$). Such a depression of thyroidal response to TSH was marked in animals injected with MMI (PTU vs MMI, $P < 0.01$).

In the second experiment, 20 animals were divided into two groups (Control and PTU 60-mg groups). Administrations of MLID, $^{131}I^-$, T_4, and measurement of thyroidal radioiodine release were the same as in the first experiment. Administration of 60 mg of PTU failed to augment thyroidal radioiodine release under T_4. With the last dose of PTU, 0.3 U of TSH was administered sc. Just before and 4 hr after TSH, blood was obtained for ^{131}I analysis. As shown in Fig. 6 (right), the increase of plasma $PB^{131}I$ was significantly less in the

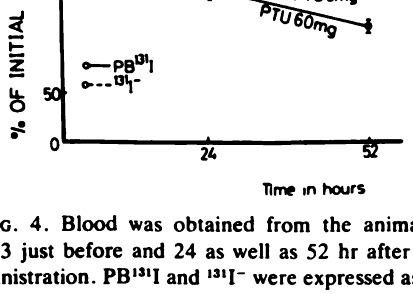

Fig. 4. Blood was obtained from the animals in Fig. 3 just before and 24 as well as 52 hr after PTU administration. $PB^{131}I$ and $^{131}I^-$ were expressed as percentage of initial plasma radioactivity. Circles and vertical lines indicated mean ± SE. Experimental procedures were the same as in Fig. 3.

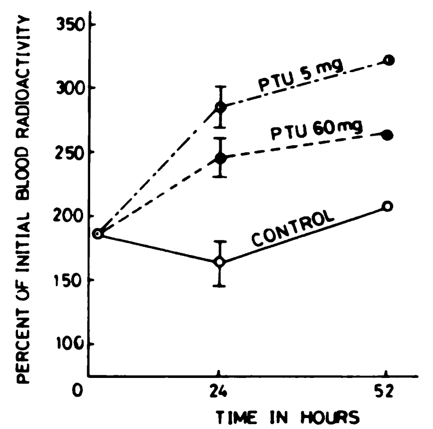

Fig. 5. TSH activity of rat plasma, as judged by mouse bioassay. Blood was obtained from rats shown in Fig. 3 just before and 24 as well as 52 hr after the administration of PTU. Plasma at 0 and 52 hr was pooled before TSH assay. Circles and vertical lines indicates mean ± SE. Statistical analysis at 24 hr: control vs PTU 5-mg, $P < 0.001$; control vs PTU 60-mg, $P < 0.01$; PTU 5-mg vs PTU 60-mg, $0.05 < P < 0.1$.

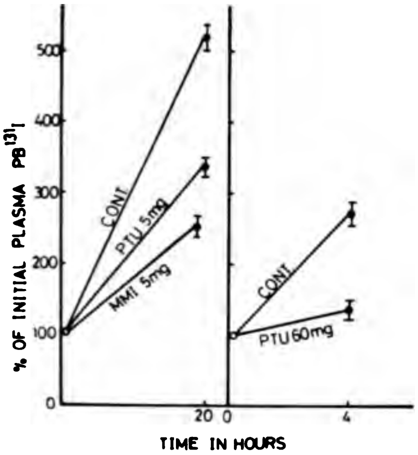

FIG. 6. On the left, 1 U of TSH was injected sc to rats treated with T_4 (15 μg) and goitrogens. Blood was obtained just before and 20 hr after TSH injection. On the right, 0.3 U of TSH was administered sc to rats treated with T_4 (15 μg) and PTU 60 mg. Blood was obtained just before and 2 hr after TSH administration. Circles and vertical lines indicated mean ± SE.

60-mg PTU group (Control vs 60-mg PTU, $P < 0.005$).

Experiment 5. Effects of graded doses of propylthiouracil or methimazole on thyroidal response to TSH in mice. Thirty mice fed MLID for 7 days were used to measure thyroidal response to TSH under the influence of goitrogens. Graded doses of PTU (0.1, 1, and 5 mg) or 5 mg of MMI in 0.5 ml of gelatin (0.5%) were administered ip 45 min before iv injection of 5 mU of TSH. Blood was obtained just before and 2 hr after TSH administration. As shown in Fig. 7, administration of TSH produced a marked increase of blood radioactivity. Administration of gelatin had no effect on thyroidal response to TSH. This increase of plasma radioactivity was augmented by 0.1 mg of PTU. However, the thyroidal response to TSH decreased progressively with increasing doses of PTU administered. MMI apparently depressed thyroidal response to TSH.

Discussion. In accordance with the previous studies (4, 5), our present data indicated that thyroidal radioiodine release increased shortly after a single injection of 1 to 5 mg of PTU into animals fed LID, and this increased rate of release continued as long as PTU injection was continued. Since PTU and MMI failed to augment thyroidal radioiodine release in rats treated with T_4, TSH was essentially linked with the manifestation of this goitrogen effect on thyroidal radioiodine release. Escobar *et al.* (4) hypothesized that PTU might cause an acute increase of pituitary TSH secretion by blocking the deiodination of T_4, a process presumably required for the manifestation of hormonal action. However, this explanation is not satisfactory, since a similar increase of thyroidal radioiodine release is also produced by moderate doses (7 to 15 mg) of MMI which is known not to block deiodination of T_4 *in vivo* (7). Simultaneous measurements of thyroidal radioiodine release, plasma $^{131}I^-$ and plasma $PB^{131}I$ indicate that a marked increase of thyroidal $^{131}I^-$ release is responsible for an increase of thyroidal radioiodine release. Two processes are undoubtedly involved in this increased release of iodide produced by goitrogens. First, goitrogens augment thyroidal radioiodine release by blocking intrathyroidal recirculation of iodide. Second, iodide release is augmented by an increased secretion of TSH produced by a reduction of thyroid hormone synthesis under goitrogen treatment. Since both processes are accentuated by increasing the doses of goitrogen admin-

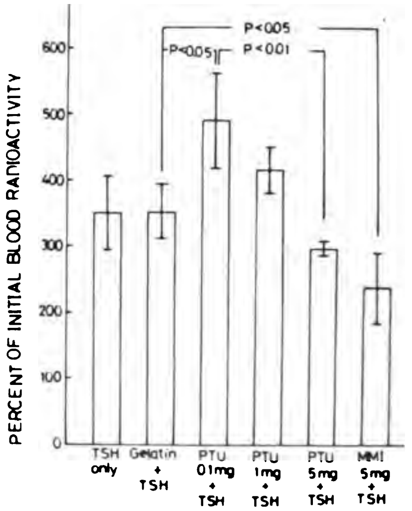

FIG. 7. Graded doses of PTU or MMI in 0.5 ml of gelatin (0.5%) were injected ip 45 min before TSH administration. Blood was obtained just before and 2 hr after TSH (5 mU) administration. gelatin + TSH vs PTU 0.1-mg + TSH, $P < 0.05$; PTU 0.1-mg + TSH vs PTU 5-mg + TSH, $P < 0.01$; gelatin + TSH vs MMI 5-mg + TSH, $P < 0.05$.

istered (1), it can be suspected that thyroidal radioiodine release is augmented with increasing doses of goitrogen administered. In contrast to this speculation, the following three unexpected findings were observed. First, when 30 mg of PTU was administered daily, an intermediate increase of thyroidal radioiodine release appeared shortly after a single injection of PTU, and this release was then followed by a final increase 30 hr later. Second, when 60 mg of PTU was administered, 34 hr elapsed before any increase of thyroidal radioiodine release was detected. Finally, when 40 mg of MMI was administered, 29 hr elapsed before any increase of thyroid secretion was detected. Since these unexpected effects of goitrogens were short-lasting, and since the final release rate was slightly faster in the 30-mg and 60-mg PTU groups than in the 5-mg group, it seemed that these findings were not simply due to general toxic actions of goitrogens.

It is possible that large doses of goitrogens depress thyroidal radioiodine release by inhibiting an increased secretion of TSH in response to goitrogens. This is unlikely, however, since a possible difference of plasma TSH between 5-mg and 60-mg PTU group is too small to account for the observed marked difference of thyroidal radioiodine release and plasma $^{131}I^-$. Also, normal increase of thyroidal radioiodine release resumes in response to this concentration of TSH after a certain period of latency. An alternative hypothesis would then be that large doses of goitrogens make the thyroid less responsive to TSH for a certain period. In order to test this hypothesis, we have measured thyroidal response to TSH in rats and mice treated with T_4. As shown in Figs. 6 and 7, large doses of PTU and MMI apparently reduced the effectiveness of TSH. It is concluded that large doses of PTU and MMI make the thyroid less responsive to TSH for a certain period through some unknown mechanism.

Summary. Thyroidal radioiodine release increased shortly after a single injection of small doses of PTU, while moderate doses of MMI produced a similar increase of thyroidal radioiodine release with a latency of 7–9 hr. Large doses of PTU and MMI failed to augment thyroidal radioiodine release for at least 29 to 34 hr after the initial administration of goitrogens, although plasma TSH increased significantly because of goitrogen administration. An increase of thyroid hormone release in response to exogenous TSH was depressed by PTU and MMI in rats and mice treated with T_4. Since this depression of TSH action only continued for a short period in spite of continuous administration of goitrogens, and since final thyroidal radioiodine release rate was similar to that produced by small doses of PTU, the effects mentioned were not simply due to general toxic action of goitrogens. It is suggested that large doses of PTU and MMI not only block thyroid hormone synthesis but also interfere with the action of TSH on thyroid hormone secretion.

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I OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 95-98 (1976)

ce for Hormonal Participation in the Natriuretic and Kaliuretic Responses to Intraventricular Hypertonic Saline and Norepinephrine[1] (39336)

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microinjection of hypertonic sodium e solution into the third ventricle atriuresis, kaliuresis, and a decline e volume in several species (1-4). esis also follows the intraventricular n of carbachol (5) or norepinephrine gesting that adrenergic and choliner- apses may participate in the pathway ed with the elicitation of central na- s and kaliuresis. The mechanism by these agents induce increased excre- monovalent cations has not been d. To determine if pituitary or hypo- ic hormones might play a role in me- these responses, lesions were placed median eminence of the tuber ciner- rats and the effects of the lesions on onse to third-ventricular injection of nic saline and norepinephrine were .

rials and methods. Male Holtzman 50-350 g) were housed communally onditions of controlled temperature hting, and were given free access to er and laboratory chow. Using ether sia, bilateral electrolytic lesions laced in the median eminence region athodal current (3 mA) passed for 17 previously described (6). Sham le- ere placed by inserting the electrodes lly to a depth of 5 mm beneath the m without passage of current. After 5 days the animals were anesthetized her and subjected to the second se- procedures (cannulation of the third ule, jugular vein, and urinary blad- previously described (4). They were r the experiments 1-2 days later. vater intake was measured in sham- d rats and those with median emi- esions.

For the experimental procedure, the animals were placed in individual metabolic cages and an infusion into the external jugular vein of 5% dextrose (0.19 ml/min) was begun. Urine samples were collected at 15-min intervals and when the rate of urine flow had reached a plateau, 2 μl of 2 *M* NaCl or 5 μg of norepinephrine bitartrate in 2 μl of 0.9% NaCl were injected into the third ventricle. This was followed by the collection of an additional six urine samples. Urine volume and Na^+ and K^+ were measured in each sample. The site and extent of the lesion was determined by microscopic examination of frozen frontal hypothalamic sections cut at 15-μm and stained with thionin.

Significance of differences between means of two groups was determined by Student's *t* test.

Results. Effect of median eminence lesions on the response to hypertonic saline. An increase in the sodium concentration of the cerebrospinal fluid (CSF) elicits a natriuretic effect in the conscious animal (4). This effect was still observed when hypertonic saline (2 μl of 2 *M* NaCl) was injected into the third ventricle in the animals with sham lesions (Fig. 1); however, destruction of the median eminence region prevented this natriuresis. Likewise, there was no increase in potassium excretion following the intraventricular injection of hypertonic saline into the rats with electrolytic lesions. The increase in CSF sodium concentration was effective in producing an antidiuresis in both groups although the degree of antidiuresis was significantly greater in the sham-operated animals.

Effect of median eminence lesions on the response to norepinephrine. Third ventricular injection of the neurotransmitter, norepinephrine, produced a natriuresis, kaliuresis, and antidiuresis in normal (4) or

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served.

sham-operated animals (Fig. 2). Median eminence lesions were effective in preventing the increases in sodium and potassium excretion and in reducing the antidiuretic response elicited by this catecholamine.

Localization of the lesions. Thionin-stained sections of the brains of rats with lesions were examined microscopically to determine the extent and the site of the damage. The rostral limit of the lesions was the optic chiasm and they extended caudally so as to destroy 1/2 to 2/3 of the median eminence. Damage to the wall of the ventricle was variable; however, in some animals, it appeared to be largely intact. There was considerable damage to the anterior part of the arcuate nucleus and variable damage to the ventromedial nucleus.

Daily water intake was increased after lesions in all animals. On the seventh postoperative day, water intake in sham-operated animals was 32.6 ± 4.3 (8)[2] ml/day, whereas in rats with median eminence lesions, it was 139.1 ± 4.0 (8) ml/day. Thus, the rats with lesions had diabetes insipidus as a result of at least partial interruption of the supraopticohypophyseal tract.

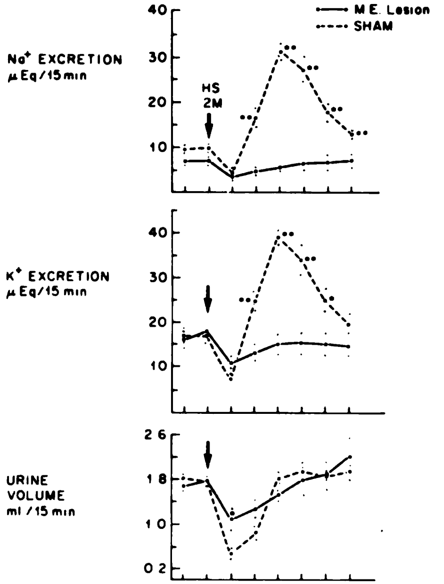

FIG. 1. Effect of median eminence lesions on the natriuresis, kaliuresis, and urine output following injection of hypertonic saline (HS) (2 *M* NaCl, 2 μl) into the third ventricle. Values for sham-operated rats are indicated by ·---·, whereas those from rats with lesions are indicated by ·—·. Standard errors are indicated by the small dots above and below each mean. * = $p < 0.05$ vs the sham-operated animals; ** = $p < 0.01$ vs sham-operated controls. *N* = 8 for the group with lesions, and 7 for the sham-operated controls.

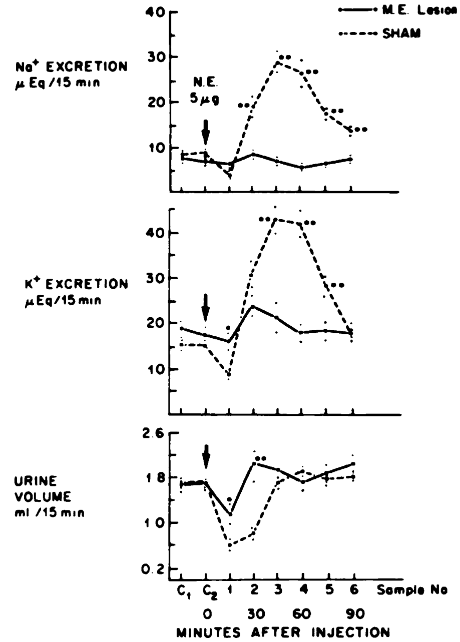

FIG. 2. Effects of median eminence lesions on the natriuresis, kaliuresis, and urine output after the injection of norepinephrine (NE) (5 μg, 2 μl) into the third ventricle. *N* = 12 for rats with lesions, and 8 for sham-operated controls.

Discussion. The results of this study clearly show that damage to the median eminence region was effective in abolishing the natriuresis and kaliuresis produced by the third ventricular injection of either hypertonic saline or norepinephrine and in significantly reducing the antidiuresis which ensued. Even though a decrease in urine volume still followed these injections in animals with lesions, there was no increase in either the excretion or concentration (not shown) of sodium or potassium ions.

Since these lesions are known to produce interference with the release of a number of pituitary hormones, it is logical to suggest that the failure to release one or more of these hormones may be responsible for the

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[2] Mean ± SE (number of rats).

e of natriuresis and kaliuresis follow-
raventricular injection of hypertonic
or norepinephrine. Previous studies
hown that lesions of this type interfere
e release of ACTH (7), growth hor-
(8), TSH (9), FSH and LH (6),
as they enhance the release of prolac-
and perhaps that of MSH (10). Intra-
cular hypertonic saline has not been
to be capable of altering release of
lenohypophyseal hormone; however,
entricular norepinephrine has been re-
l to stimulate gonadotropin and possi-
olactin release (see 11 for review of
erature). Neither of these hormones
en shown to be natriuretic in the rat.
ddition to producing these alterations
enohypophyseal hormone secretion,
lesions by their interruption of the
pticohypophyseal tract also interfere
he release of both oxytocin (12) and
essin (13).

evidence for interruption of the su-
ticohypophyseal tract was the pres-
f diabetes insipidus in all of the rats
sions. We have previously shown that
lesions block suckling-induced re-
f oxytocin (12). Even though the ani-
ad diabetes insipidus, the urinary out-
llowing infusion of 5% glucose solu-
as similar in animals with lesions and
sham-operated controls. Presumably,
as because of the induction of a water
is in response to the infusions. Thus,
gh the absence of these responses fol-
the lesions suggests hormonal partic-
, there is a wide range of possibilities
which hormone or hormones may be
ed in mediating the natriuretic re-
s in the intact animal. Antidiuretic
ne (ADH), oxytocin, and melanocyte
ating hormone (MSH) are all natri-
under the conditions of a water di-
similar to those employed in the pres-
periment (14, 15).

H does not appear to be essential for
oduction of central natriuresis since it
curred in animals with hereditary dia-
nsipidus and the complete absence of
(16). Increases in the blood levels of
have been observed following intra-
cular injection of norepinephrine in
t (17) and the direct application of
hypertonic saline or norepinephrine to the supraoptic nucleus of the cat (18). In view of the retention of the natriuretic response in the rat with hereditary diabetes insipidus and the fact that large doses of pitressin did not influence the urinary response to intraventricular hypertonic saline (17), it appears that ADH does not play an essential role in central natiuresis.

It is quite possible that an increased release of oxytocin, MSH and/or the proposed natriuretic hormone (19) may be involved in the mediation of central natriuretic effects; however, the natriuretic response to intraventricular carbachol persisted in hypophysectomized rats which should be completely deficient in MSH and largely deficient in oxytocin (5). Thus, the results are most consistent with mediation of the natriuretic response by a natriuretic hormone (19).

The possibility that the lesions may interrupt a neural pathway distinct from their effects on pituitary hormonal secretion cannot be ruled out. Central natriuresis has also been explained on the basis of alterations in blood pressure, glomerular filtration rate, or renal neural input; however, we have previously reported a lack of correlation between blood pressure changes and the natriuretic effects of hypertonic saline and norepinephrine (20).

Summary. To examine if hypothalamic or pituitary hormones are involved in the induction of the natriuresis which follows the injection of hypertonic saline or norepinephrine into the third ventricle, lesions were placed in the median eminence and the responses to intraventricular norepinephrine or hypertonic saline were evaluated. Sham lesions in which the electrode was lowered into the brain but stopped short of the hypothalamic region did not interfere with the natriuresis, kaliuresis, and antidiuresis induced by the third ventricular injection of either hypertonic sodium chloride or norepinephrine. Lesions in the median eminence which induced diabetes insipidus as evidenced by an increase in water consumption to approximately four times normal completely abolished the natriuresis and kaliuresis in response to intraventricular hypertonic saline or norepinephrine and diminished the antidiuresis. The observations suggest the possibility that a natriuretic hormone(s) is involved in the induction of central natriuresis.

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Adrenergic Regulation of the Secretion of an Anticomplementary Factor in Mouse Saliva[1] (39337)

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(Introduced by D. M. Woodbury)

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otent inhibitor of complement activity een detected in mouse saliva in the ird controls employed in a microcom- nt fixation assay for nerve growth fac- , 2). Such salivary anticomplementary y might have physiological signifi- since complement has been demon- d in both normal and inflamed gingi- sues (3) and in the crevicular fluid of ts with periodontal disease (4) or in- d gingiva (5). In addition, anticomple- ry factors have been detected in hu- aliva (6, 7), and it has been suggested ich factors could inhibit complement- ted processes in diseased gingiva (6). retion of the anticomplementary fac- uld be governed by either the sympa- or parasympathetic nervous system. elicited in response to parasympa- stimulation has a different protein sition from that secreted in response npathetic stimulation (8, 9). In order ermine which branch of the autonomic us system regulates secretion of the mplementary factor, saliva elicited by sympathomimetic and parasympa- metic agents was analyzed for anti- ementary activity. In addition, other acological methods were employed to lly characterize the anticomplemen- actor.

erials and methods. Saliva was col- from ten- to sixteen-week-old male The animals were anesthetized with barbital, 60 mg/kg. Salivation was in- by the intraperitoneal (ip) adminis- n of a secretagogue. The secreta- s and the range of doses for each were: pilocarpine, 0.1 to 0.8 mg/kg; epinephrine, 2.0 to 6.6 mg/kg; norepinephrine, 0.7 to 2.5 mg/kg; and isoproterenol, 1.0 to 5.0 mg/kg. In a few cases, about one-tenth the ip dose of secretagogue was injected under the sheath of connective tissue covering the submaxillary gland. The results were comparable to those after ip injection. In all experiments, saliva was collected in a microcapillary tube placed between the tongue and the floor of the mouth. In some cases, small aliquots of saliva were collected sequentially from a single animal given one dose of a secretagogue. In other cases, all of the saliva elicited by one dose of secretagogue was collected as one sample. Immediately after collection, the saliva was frozen at −40° until the assays were performed.

Saliva, trypsin, chymotrypsin, thrombin, Kallikrein, γ-subunit of 7S nerve growth factor (10), amylase, carbonic anhydrase, and carboxypeptidase A were tested for anticomplementary activity. This was determined following the basic procedure of Moore and Perez (11) as modified from Wasserman and Levine (12). Samples of saliva or of an enzyme preparation were diluted in sodium veronal buffer (5 mM, pH = 7.5) containing 0.1% gelatin and 0.005% Triton X-100. A 5-μl aliquot of each dilution was added to 50 μl of complement-containing guinea pig serum diluted 1:320 in the same buffer. The resultant mixture was incubated at 4° for 18–20 hr. After the incubation, 0.3 ml of a suspension of sheep red blood cells sensitized by treatment with hemolysin was added to each reaction mixture. The samples were incubated for 60 min at 38° to allow complement-mediated hemolysis and then centrifuged to pellet the red blood cells (12,000 *g* for 10 min). The absorbance of the supernatant at 413 nm was determined. The optical density of the blanks which have 5 μl of buffer added in

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place of the sample was usually close to 1.0. This parameter was initially set by adjusting the amount of complement in the reaction.

In order to quantify salivary anticomplementary activity, a series of dilutions of a saliva sample was assayed as described above. The dilution at which the resultant optical density was one-half that of the blanks (i.e., 50% inhibition of complement-mediated hemolysis) was determined. The reciprocal of this dilution will be defined as the number of anticomplementary units (ACU) in 5 μl of the undiluted saliva sample. Since the highest concentration of saliva examined was a 1:10 dilution, salivary anticomplementary activities of less than 10 ACU were not quantified.

In studies involving protease inhibitors, a 5-μl aliquot of inhibitor (780 ng/μl) was added to the complement-containing solution in addition to 5 μl of the diluted saliva or enzyme sample. The inhibitors studied were aprotinin, Kunitz pancreatic trypsin inhibitor, soybean trypsin inhibitor, ovomucoid trypsin inhibitor, and lima bean trypsin inhibitor.

Protein determinations were made using the method of Lowry *et al.* (13) with bovine serum albumin as a standard.

L-epinephrine bitartrate, trypsin (bovine, type III, 12,500 *N*-benzoyl-L-arginine ethyl ester units/mg), α-chymotrypsin (bovine, type II, 50 benzoyl-L-tyrosine ethyl ester units/mg), thrombin (bovine, grade I, 250 NIH units/mg), α-amylase (ovine, type I-A, 2,250 Wilbur-Anderson units/mg), carbonic anhydrase (bovine, 41 hippuryl-L-phenylalanine units/mg), trypsin inhibitor from beef pancreas (1 mg inhibits 3.1 mg trypsin), trypsin inhibitor from soybean (1 mg inhibits 1.4 mg trypsin), trypsin inhibitor from ovomucoid (1 mg inhibits 0.9 mg trypsin), trypsin inhibitor from lima bean (1 mg inhibits 4 mg trypsin), and bovine serum albumin (fraction V, 96–99%) were purchased from Sigma Chemical Co., St. Louis, Mo. Pilocarpine hydrochloride was purchased from Merck Chemical Co., Rahway, N.J., noradrenaline hydrochloride and isoproterenol hydrochloride from Regis Biochemicals, Morton Grove, Ill., and aprotinin (Trasylol, 10,000 kallikrein inhibition units/ml) from FBA Pharmaceuticals, New York, N.Y. Kallikrein (a registered trademark of Bayer AG, 1290 kallikrein units/mg) was kindly supplied by Drs. Elder and Schmidt-Kastner, Bayer A. G., Wuppertal, Germany. The γ-subunit of 7S nerve growth factor (10) was a generous gift from Dr. E. M. Shooter of Stanford University.

Results. The protein concentration, anticomplementary factor concentration, and flow rate of the saliva were measured at different times following single injections of either pilocarpine (Fig. 1) or epinephrine (Fig. 2). When epinephrine rather than pilocarpine was used, the results showed several marked contrasts: (a) the flow rates were lower, (b) the protein concentrations were approx 50-fold greater, and (c) the anticomplementary factor concentrations were more than 1000-fold greater. A change in protein concentration from one aliquot to the next appeared to be accompanied by a parallel shift in anticomplementary factor levels in

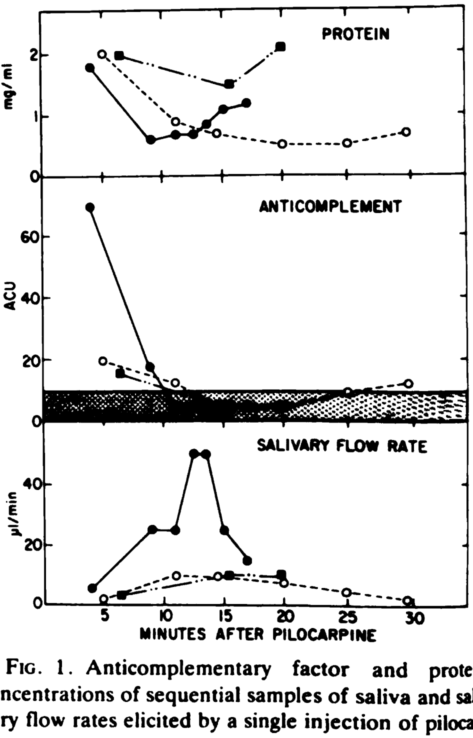
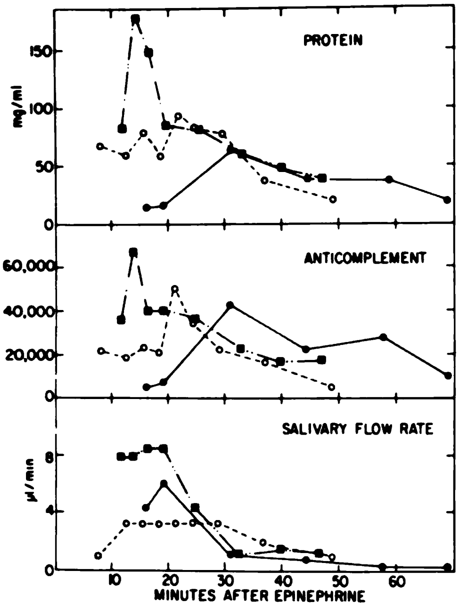


FIG. 1. Anticomplementary factor and protein concentrations of sequential samples of saliva and salivary flow rates elicited by a single injection of pilocarpine. Each set of like symbols represents data from a single animal. Values in the stippled area of the anticomplement panel represent estimates since activities of less than 10 ACU were not quantified (see Materials and Methods).



G. 2. Anticomplementary factor and protein entrations of sequential samples of saliva and sali- flow rates elicited by a single injection of epineph- Each set of like symbols represents data from a e animal.

epinephrine-induced saliva, but not in pilocarpine-induced saliva.

he relationship between protein concen- on and anticomplementary factor con- ration with isoproterenol and norepi- hrine as secretagogues was examined in :r to determine whether the epineph- -induced secretion was due to action on lrenergic or β-adrenergic receptors. se data along with the corresponding i from the epinephrine treatment are vn in Fig. 3. An estimate of the slopes intercepts of these curves was obtained g linear regression analysis. These esti- es of the slopes and their standard errors shown in Table I.

nce salivary anticomplementary activity it be due to the action of salivary pro- ytic enzymes on the complement pro- ;, several proteolytic enzymes thought e present in saliva (14, 15) were exam- for anticomplementary activity. The in Fig. 4 demonstrate the anticomple- tary activity of trypsin, chymotrypsin, nbin, and Kallikrein, and the lack of ity of the esteroproteolytic γ-subunit of 7S nerve growth factor (16). Amylase, carbonic anhydrase, and carboxypeptidase A were also studied but lacked anticomplementary activity (data not shown).

Salivary anticomplementary activity was examined in the presence of protease inhibitors in order to determine whether the anticomplementary factor might be a proteolytic enzyme (Fig. 5). The salivary anticomplementary factor was inhibited by aprotinin and by Kunitz pancreatic trypsin inhibitor

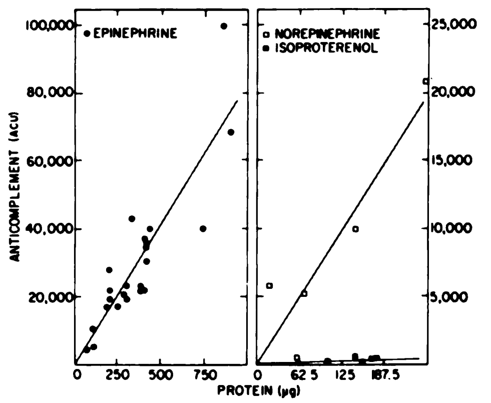


FIG. 3. Correlation between anticomplementary factor and protein in secretions elicited by adrenergic agents. The lines were determined by linear regression analysis. The correlation coefficients for epinephrine, norepinephrine, and isoproterenol were 0.88, 0.91, and 0.28, respectively.

TABLE I. SPECIFIC ACTIVITIES OF ANTICOMPLEMENTARY FACTOR IN SECRETIONS ELICITED BY VARIOUS AUTONOMIC AGENTS.[a]

Agonist	Salivary anticomplementary activity (ACU/μg protein)
Pilocarpine [cholinergic]	0.3 ± 0.1 (10)
Isoproterenol [β-adrenergic]	1.8 ± 3.6 (5)
Epinephrine [α + β-adrenergic]	85. ± 10. (24)
Norepinephrine [α-adrenergic]	78. ± 20. (5)

[a] Values are the slopes of linear regression lines ± the standard error of the slope. The regression lines for all data groups except pilocarpine are shown in Fig. 3. Number of samples is shown in parentheses. The receptors primarily stimulated by each agonist are indicated in square brackets. The following are statistically significant ($P < 0.01$): (a) pilocarpine vs both epinephrine and norepinephrine, and (b) isoproterenol vs both epinephrine and norepinephrine.

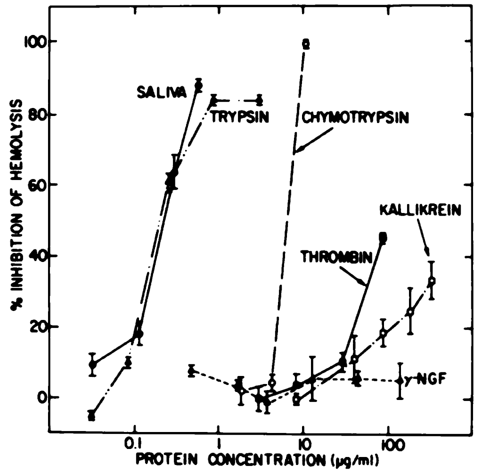


FIG. 4. Anticomplementary activity of proteolytic enzymes and saliva. Concentrations are final concentrations in the 55-μl reaction mixture. Saliva values are for total salivary protein concentrations in the 55-μl reaction mixture. Bars indicate standard errors of the means. Each point represents the mean of three to six samples.

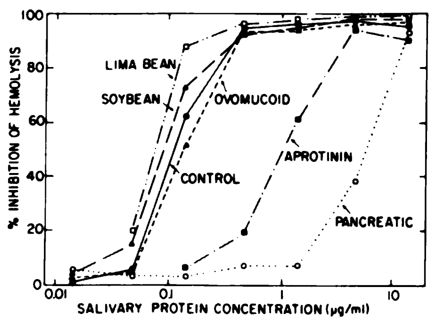


FIG. 5. Effects of protease inhibitors on salivary anticomplementary activity. The results presented were obtained using epinephrine-induced saliva from one animal. Each inhibitor has been examined with at least three different saliva samples and the same inhibition pattern was always obtained. At each concentration of salivary protein, either a 5-μl aliquot containing 3.9 μg of inhibitor or a 5-μl aliquot of buffer was added.

but not by soybean trypsin inhibitor, ovomucoid trypsin inhibitor, or lima bean trypsin inhibitor.

The ability of these protease inhibitors to block the anticomplementary activity of trypsin, chymotrypsin, and Kallikrein was examined in order to compare pharmacologically these enzymes with the salivary anticomplementary factor (Table II). The spectrum of inhibition of salivary anticomplementary activity was the same as that of Kallikrein but unlike those of trypsin or chymotrypsin.

Discussion. The present study demonstrates that mouse saliva contains one or more potent anticomplementary factors. The concentration of this factor was more than 1000 times greater in secretions elicited by epinephrine, a sympathomimetic agent, than in those induced by pilocarpine, a parasympathomimetic agent (Figs. 1 and 2). These results also show that pilocarpine elicits a rapid flow of a secretion with relatively low protein content, while epinephrine induces a slower flow of a secretion with a 50-fold higher protein content. These observations on flow rate and protein content are consistent with results obtained by other investigators (14, 17, 18).

Since both anticomplementary factor and total protein concentrations were higher in the epinephrine- than in the pilocarpine-induced secretions, specific activities were determined to compare the activity per unit protein (Table I). The mean specific activity of epinephrine-induced saliva was 283 times that of pilocarpine-induced saliva. Since high doses of pilocarpine (10 mg/kg ip) produce an indirect sympathomimetic effect in rat salivary glands (19), even the low level of anticomplimentary activity found in the pilocarpine-induced saliva might be due to this indirect adrenergic action. These data imply that secretion of the anticomplementary factor is primarily regulated by sympathetic activity.

The levels of salivary anticomplementary factor were also determined following an α- and a β-adrenergic agonist in order to characterize the adrenergic receptor involved.

TABLE II. EFFECTS OF PROTEASE INHIBITORS ON ANTICOMPLEMENTARY ACTIVITY[a].

Protease inhibitor	Anticomplementary activity			
	Trypsin	Chymotrypsin	Kallikrein	Saliva
Soybean	+	+	–	–
Lima bean	+	+	–	–
Ovomucoid	+	–	–	–
Pancreatic	+	+	+	+
Aprotinin	+	+	+	+

[a] A plus indicates a greater than 75% inhibition of anticomplementary activity, and a minus indicates a less than 15% inhibition.

α-adrenergic agonist norepinephrine d saliva having a mean specific activity nes that of the β-adrenergic agonist terenol and 260 times that of the cho- ic agonist pilocarpine (Table I). In ad- ı, the specific activity of norepineph- nduced saliva was not significantly dif- t from that elicited by epinephrine, ı possesses both α- and β-adrenergic ty (Table I). Thus, release of the sali- anticomplementary factor appears to imarily regulated through the action of mpathetic nervous system on α-adre- receptors within the salivary glands.

r observation that salivary anticomple- ry activity can be inhibited by both inin and Kunitz pancreatic trypsin in- r strongly suggests that this activity be due to a salivary protease. Such a ry anticomplementary protease would o be capable of selectively inactivating r more of the complement components presence of a great excess of other ins. (At 50% hemolysis, complement ins only account for 0.06% of the total in.)

eral highly purified proteases have examined for anticomplementary ac- in order to compare them to the sali- actor (Fig. 4). Expressed in terms of salivary protein, the potency of the ry anticomplementary factor was to that of recrystallized trypsin, the potent enzyme examined. Thus, the ry anticomplementary factor must be lerably more potent than any enzyme l since it probably constitutes only a proportion of total salivary protein.

effects of five protease inhibitors been determined on the anticomple- ry activity of trypsin, chymotrypsin, allikrein. The results show that only inhibitors which have been reported ck the proteolytic activity of a given e (20) will inhibit its anticomplemen- ctivity (Table II). This strongly sug- that the anticomplementary activity of f these enzymes is correlated with its lytic activity. To our knowledge this first demonstration that kallikrein pos- anticomplementary activity. This be quite significant since kallikrein is hought to act on a limited number of n substrates.

The inhibition spectrum of the salivary anticomplementary factor indicates that it might be a kallikrein-like enzyme (Table II). Kallikrein is present in both animal and human saliva (21, 22) and appears to be selectively secreted through α-adrenergic stimulation (21, 23). However, the Kallikrein from ovine pancreas used in this study differed from the salivary anticomplementary factor both in potency and the slope of the dose-response curves (Fig. 4). Since others have observed marked differences between organ and salivary kallikrein (24), these observations do not demonstrate that the salivary anticomplementary factor is not salivary kallikrein. Final resolution of this question must await purification and isolation of the salivary anticomplementary factor.

Summary. Mouse saliva contains a potent inhibitor of complement activity. The secretion of this inhibitor appears to be regulated by action on α-adrenergic receptors for two reasons. First, an α-agonist (norepinephrine) elicited saliva with a 260-fold higher specific activity of the inhibitor than that obtained with a cholinergic agent (pilocarpine). Second, the α-agonist elicited saliva having a 43-fold greater specific activity than that obtained following administration of a β-adrenergic agonist (isoproterenol). This anticomplementary factor probably proteolytically degrades one or more of the complement components since it is inhibited by several protease inhibitors. The salivary anticomplementary factor is more potent than trypsin, chymotrypsin, thrombin, or Kallikrein. The anticomplementary factor has a pattern of inhibition like that of Kallikrein but unlike those of trypsin or chymotrypsin.

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physeal Cartilage Cyclic Nucleotide Phosphodiesterase: Partial Purification and Kinetic Characteristics of Multiple Enzymes (39338)

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tiple forms of 3′,5′-cyclic AMP phos- esterase (PDE) have been demon- l in many tissues (1-13), and these es may have important and different tory functions within cells. Previous from our laboratory has identified AMP PDE activity in chicken epiphy- nd articular cartilage (14). We have emonstrated the inhibition of the low ecies of these enzymes by L-triiodothy- (T-3) and several nonsteroidal anti- mmatory agents (14, 15).

determine the physiological signifi- of PDE inhibition by these pharmaco- l agents and to relate the inhibition to gulatory mechanisms, it was necessary racterize the multiple molecular forms E present in cartilage. In this paper, scribe the presence of multiple molec- orms of PDE in epiphyseal cartilage, ng by their affinity for cyclic AMP and GMP, their inhibition by triiodothyro- T-3) and indomethacin, and their sep- n on DEAE Bio-Gel A column chro- raphy.

erials and methods. Ammonium salts lic [G-^{3}H]AMP (24.0 Ci/mmole) and lic [G-^{3}H]GMP (3.46 Ci/mmole) were ased from New England Nuclear. The ctive compounds were purified by yer chromatography before use as fol- A 50% ethanol solution of either nucleotide was applied to an Eastman atogram cellulose sheet (13255) as a (10-15 μl per cm), and the chro- raph was developed with isopro- -conc. NH_4OH-water (7:1:2, v/v) for The R_f values for cyclic AMP and GMP in this system were 0.56-0.60 .32-0.36, respectively. After drying hromatogram, the cyclic nucleotide vas scraped off, and the nucleotide ex- d with water at 5°C for 2 to 3 hr. After fugation to clear the radioactive solution, the cyclic nucleotide solutions were stored at −20°.

Snake venom *(Ophiophagus hannah)* and cyclic GMP were obtained from Sigma, and cyclic AMP (free acid, monohydrate) was from P-L Biochemicals, Inc. DEAE Bio-Gel A (100-200 mesh) was obtained from Bio-Rad Laboratories and was equilibrated with the column buffer prior to use. Anion exchange resin (Bio-Rad AG1-X2, 200 to 400 mesh) was washed in succession with 0.5 *N* NaOH, H_2O, 0.5 *N* HCl and H_2O until neutral. Indomethacin was provided by Dr. T. Y. Shen of the Merck Institute for Therapeutic Research, and T-3 (3,3′,5-triiodo-L-thyronine) was obtained from Schwarz/Mann.

Phosphodiesterase activity was assayed by a procedure modified from the method of Thompson and Appleman (16, 17). The reaction mixture was identical to that described by them (16, 17) except that it contained 0.1 ml of snake venom. Reactions were initiated by addition of the PDE preparation, and the mixture was incubated for 10 min at 30°. Reactions were stopped by the addition of 0.5 ml of cold absolute ethanol and chilled in an ice-water bath. The reaction mixture was then transferred onto a small anion exchange column (5 × 30 mm) which was prepared from 1.5 ml of 1:2 slurry of AG1-X2 resin in a Pasteur pipet (7 × 146 mm). The reaction tube and the column were washed successively with 3 × 1 ml of water, allowing each wash to sink into the resin before adding the next one. An aliquot of 1 ml out of 4 ml of the combined effluent and washings was counted in 10 ml of scintillation fluid. Enzyme activity was linear with respect to time, protein concentration, and up to 20% conversion of substrate to product under the conditions of the assay as described. Counting of ^{3}H was carried out with the Model 3310 Packard Tri-

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Carb liquid scintillation spectrometer. The scintillant solution was a modified Bray's solution (18) which contained 60 g of naphthalene, 4 g of 2,5-diphenyloxazole, 200 mg of 1,4-di-2-(5-phenyloxazolyl) benzene in 100 ml of methanol, and 900 ml of 1,4-dioxane. When counted in 10 ml of liquid scintilator, the efficiency of this method for tritium was 20% in the presence of 1.0 ml of tritiated water.

One unit of PDE activity was defined as the amount of enzyme required to hydrolyze 1 pmole of cyclic mononucleotide per min at 30°. Specific activity was expressed as enzyme units per milligram of protein.

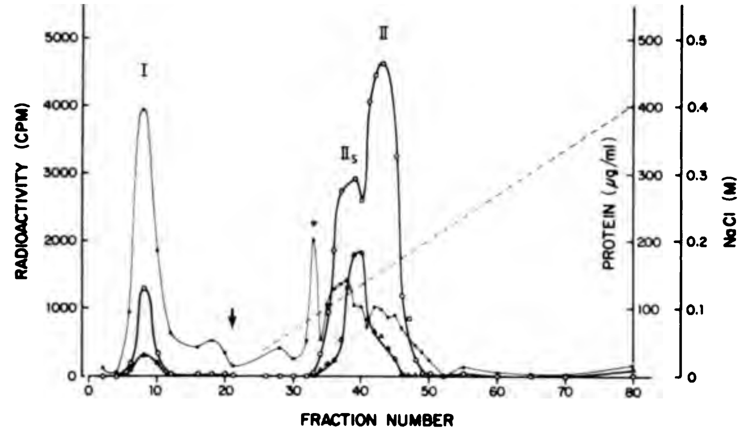
Upper epiphyseal cartilage zones removed from 8-week-old hybrid Hubbard broiler chickens by the method of Wuthier (19) as previously described (14) were used as the enzyme source. The fine shavings from four chickens were homogenized in 48 ml of ice-cold 0.05 *M* Tris-Cl buffer (pH 8.0) containing 0.25 *M* sucrose, and then centrifuged at 100,000 *g* for 90 min. The slightly reddish clear supernate solution, containing approx 2.7 mg of protein per ml, was used as the source of the crude soluble enzyme. The precipitate from the 100,000 *g* centrifugation was rehomogenized and centrifuged at 100,000 *g* thrice more, and finally suspended in the homogenizing solution to a volume equal to one-third the first supernatant solution. The soluble and particulate fraction could be stored at −20° for at least several weeks without loss of activity. Protein determinations were carried out by the Lowry procedure (20) with the use of crystalline bovine serum albumin as a standard. For column fractions in which 2-mercaptoethanol was present, a standard curve was prepared in the presence of the column buffer.

The DEAE Bio-Gel A column (1.3 × 22 cm) used for enzyme purification was equilibrated with 0.05 *M* Tris-Cl buffer (pH 8.0) containing 3.75 m*M* 2-mercaptoethanol. A portion of the soluble enzyme (30 mg of protein) dialyzed overnight against the column buffer, was applied to the column, and then washed with the same buffer until the A_{280} in the washing returned to a base line level. The column was then eluted with a linear gradient generated from 150 ml of the column buffer and 150 ml of the buffer containing 0.4 *M* NaCl, and 5-ml fractions were collected. The column flow rate was approx 12 ml/hr. One-tenth milliliter of the enzyme solution was used to assay for cyclic AMP PDE activity and 0.2 ml for cyclic GMP PDE activity. Substrate concentration was 1 μM in both cases.

Enzyme control incubations included a no enzyme reaction mixture, appropriate solvent concentrations (2% v/v DMSO) to account for any inhibition due to the solvent used with triiodothyronine and indomethacin and incubations to establish that the observed inhibition of PDE activity was not the result of the inhibition of 5'-nucleotidase activity. The assay for 5'-nucleotidase activity was performed using the same reaction mixture as described for the PDE assay except for the use of 5'-AMP as the substrate instead of cyclic AMP and snake venom was omitted. I_{50} values were obtained by plotting the logarithm of inhibitor concentration as a function of the percentage of enzyme inhibition. To determine the apparent K_m and V of the partially purified enzymes, substrate concentrations between 0.8 and 16 μM were used and the specific radioactivities of the substrate, cyclic [^{3}H]AMP, and cyclic [^{3}H]GMP, were 52,500 cpm/nmole and 47,500 cpm/nmole, respectively. Nonlinear Lineweaver-Burk plots for chicken epiphyseal cartilage PDE have been shown previously (14); the present investigations only defined low K_m values since they are probably more important with respect to the physiological activity of the enzyme.

Results. Separation of cyclic 3',5'-nucleotide phosphodiesterase activities by column chromatography. The high speed supernatant solution of chicken epiphyseal cartilage extract was fractionated by DEAE Bio-Gel A column chromatography, and the fractions were analyzed for cyclic mononucleotide hydrolyzing activity, 5'-nucleotidase activity, and protein content (Fig. 1).

Despite the use of a different ion exchange column (DEAE-cellulose) and various elution procedures, the cyclic AMP PDE activities in the major peak (II_s and II) could not be resolved into two separate peaks. As shown in Fig. 1, peak I utilized both cyclic AMP and GMP as substrates; the activity towards cyclic GMP was much



G. 1. Separation of multiple forms of PDE from chicken cartilage extract. Unabsorbed protein was washed nd then (↓) a linear NaCl gradient (- - -) up to 0.4 M was applied. Fractions of 5 ml were collected and their ic AMP-PDE (○) and cyclic GMP-PDE (△) activities as well as their protein content (●) were monitored. ioactivities found in 1-ml aliquots from 4-ml filtrate (see the text) were shown. The nucleotidase-activity peak found in fraction 33 (*).

TABLE I. THE EFFECT OF INDOMETHACIN AND L-TRIIODOTHYRONINE ON PURIFIED PDE FRACTIONS.[a]

Chromatographic fraction	I_{50} values (M)	
	Indomethacin	T-3
Peak I	1.7×10^{-3}	1.8×10^{-4}
Peak II$_s$	2.5×10^{-5}	1.0×10^{-5}
Peak II	1.1×10^{-4}	3.0×10^{-6}

[a] I_{50} values were obtained using 1 μM cyclic AMP as the substrate.

er than that for cyclic AMP. The major k appeared also to utilize both cyclic leotides as substrates; however, the en-ne activity towards cyclic GMP disapred from the fractions obtained after the ximal cyclic AMP-PDE activity was ated. These findings suggested that two ms of PDE existed, one associated with shoulder (II$_s$) which utilized both cyclic leotides, and the other associated with slowest eluting peak (II) which utilized y cyclic AMP as substrate. As compared he specific activity of the crude epiphyl enzyme with cyclic AMP as the subite, peak I specific activity was increased a factor of 1.5, the apex of the peak II$_s$ increased by 10.5 and peak II (fractions n the downslope) by 20-fold. The peak oted by the asterisk (Fig. 1) contained he 5'-nucleotidase activity present in the mn eluant.

inetic characteristics of the PDE chroographic fractions. Peak I, which repreted a single homogeneous peak, had an arent K_m of 5 μM with cyclic AMP as strate and a V of 36 units ml^{-1}. The low vity of this enzyme with cyclic GMP as strate obviated the determination of e kinetic parameters with that substrate. k II$_s$ had an apparent K_m of 1.2 μM with ic AMP as substrate and a V of 38 units $^{-1}$. Peak II, using fractions without cyclic GMP activity, had an apparent K_m of 1.2 μM and a V of 45 units ml^{-1}. Kinetic analyses of the fraction with highest activity towards cyclic GMP in peak II$_s$ gave an apparent K_m of 1.2 μM for cyclic GMP as substrate and a V of 29 units ml^{-1}.

Inhibition of purified PDE by indomethacin and L-triiodothyronine. Varying the inhibitor concentrations and determining the percentage of normal (no inhibitor) enzyme activities in a soluble and particulate enzyme preparation gave relatively high I_{50} values and demonstrated no particular preference for the inhibition of the soluble or particulate enzyme species by indomethacin or T-3. Under these conditions, the I_{50} values for these inhibitors showed relatively similar capacities to inhibit these enzyme species.

The effect of these inhibitors on various soluble enzyme fractions purified by column chromatography are shown in Table I. A comparison of the I_{50} for indomethacin and T-3 obtained from various enzyme fractions after DEAE Bio-Gel A chromatography demonstrated differences in the capacity of indomethacin and T-3 to inhibit these enzyme fractions.

Discussion. Since work from our laboratory has shown that triiodothyronine (T-3), the most potent thyroid hormone, and indomethacin, a moderately potent anti-inflammatory agent inhibit crude epiphyseal cartilage cyclic nucleotide phosphodiesterase, the present studies were undertaken to purify epiphyseal cartilage PDE and to test various purified fractions for their inhibition by these agents.

The modified PDE assay, as described herein, avoided the high values for the enzyme blanks caused by the heat step in the original Appleman assay (16, 17). The use of cold ethanol to terminate the one-step assay is a simple and adequate method and is somewhat easier to perform than the method proposed by Appleman for his revised assay (21). An additional difficulty was encountered when the original PDE assay (16, 17) was used to assay purified fractions after ion exchange chromatography. The presence of sodium acetate or sodium chloride resulted in an increase in the enzyme blank. This is caused by the diminished capacity of the batch treatment of the reaction mixture with anion exchange resin to remove PDE substrates. The modified assay did not give high blank values in the presence of salts, had a low blank value, and permitted the assay of PDE activity at low enzyme concentrations. A further advantage of this assay on the PDE isolated from epiphyseal cartilage was the presence of significant 5′-nucleotidase activity in the crude enzyme. Approximately 90% of the 5′-adenosine monophosphate formed in the reaction was converted to adenosine by 0.3 mg of crude cartilage enzyme in the absence of snake venom (5′-nucleotidase). Thus, a one-step assay with 5′-nucleotidase in the reaction mixture from the outset was more advantageous for our work than the two-step Appleman procedure.

Analyses of a carefully washed 100,000 *g* sedimented enzyme fraction demonstrated PDE activity; therefore as has been shown for other tissues (22), epiphyseal cartilage contained a particulate and soluble PDE fraction. The particulate enzyme utilized both cyclic AMP and GMP as substrates. The relative percentages of PDE activity in the soluble and particulate fractions with 1 μM cyclic AMP as substrate were 86.5 and 13.5%, respectively. With cyclic GMP (1 μM) as substrate the relative percentage of the soluble and particulate fractions were 93.5 and 6.5%, respectively.

The I_{50} values determined for indomethacin and T-3 showed that peak I was inhibited by these agents at relatively high concentrations (Table I). These high values probably indicate that no physiological significance should be given to the effect of these agents on peak I enzyme activity. The ratio of the I_{50} values obtained with indomethacin and enzyme fractions from peaks II_s and II showed a 4.4-fold difference. Similar data were obtained by comparing T-3 I_{50} values from peak II_s and II. Since the I_{50} value for T-3 was lowest with peak II fractions and the I_{50} value for indomethacin was lowest for peak II_s, it might be suggested that there is a difference in drug sensitivity between the enzyme activities of II and II_s.

Although by no means conclusive proof, the substrate specificities and the inhibitor activities suggest that the major PDE peak (II_s and II) probably represents two separate enzyme species. The literature supports such a conclusion since many workers have now described three peaks of PDE activity in various tissues (17, 23–26).

The concentration of indomethacin resulting in PDE inhibition is within the dosage range of this drug in clinical practice. The fact that this agent is an inhibitor of a low K_m species of PDE, the proposed physiologically active enzyme, permits one to consider the pharmacological effect(s) of this anti-inflammatory drug on cyclic nucleotide metabolism in epiphyseal cartilage.

Even though the concentrations of T-3 inhibiting PDE activity are not within the physiological range, we (15) and others (24–35) have addressed this question of comparing *in vitro* hormone effects with *in vivo* action. Since thyroid hormone does effect bone growth and maturation *in vivo*, the effect of T-3 on the enzymes involved in

nucleotide catabolism suggests to us
ɔne of the regulatory effects of this
one may be mediated via the cyclic
otide system.

ce PDE activity probably has a role in
:gulation of cell growth and prolifera-
36), manipulation of specific PDE en-
(s) by such inhibitors may represent an
ıach to altering certain pathological
sses such as premature epiphyseal clo-
Subsequent studies may provide corre-
ıs between cell structure and enzyme
ion.

ım a basic point of view, the PDE in-
ırs, triiodothyronine and indometha-
nay be useful in the investigation of
heterogeneity in other tissues.

nmary. Partial purification of chicken
ıyseal PDE activity by centrifugation
column chromatography has defined
distinct peaks of PDE activity. The
· eluting peak (I) has a higher apparent
ır cyclic AMP than the slower eluting
r peak (II_s and II). Peak I has greater
ty towards cyclic AMP as a substrate
towards cyclic GMP but does use both
rates. Peak I is not inhibited by T-3 or
ıethacin at physiological concentra-
Substrates studies demonstrate the
nce of at least two overlapping PDE
:s in the major peak (II_s and II). There
gestive evidence that indomethacin is a
potent inhibitor of peak II_s which can
ither cyclic AMP or cyclic GMP as
'ates, whereas T-3 is a more potent
tor of fractions eluting where the en-
only has activity with cyclic AMP.

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ailure of Ovalbumin Associated with Allogeneic Spleen Cells to Stimulate Proliferation of Lymph Node Cells of Immune Mice[1] (39339)

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specificity of cytotoxic T-cells for an-
nodified target cells has been shown
ude the H-2 antigen of the cell used to
ate activation of the effector cells

Using the incorporation of [^{3}H]-
dine as an assay of lymphocyte blasto-
s, Rosenthal and Shevach have shown
guinea pigs proliferation of immune
ocytes induced by PPD bound to
phages was much greater if the macro-
s were syngeneic to the responding
han if they were allogeneic (7). A
r requirement of syngeneic macro-
s has been more difficult to demon-
in mice because of the inhibitory
of mouse macrophages on cell prolif-
n and because of the higher back-
d produced by a much more prompt
lymphocyte reaction in this specie.

e experiments reported here we have
ly circumvented the inhibitory effect
use macrophages by reducing their
r and largely eliminated the mixed
ocyte reaction by using F$_1$ mice made
ric with bone marrow from one of the
al strains. Using lymph node cells of
ized chimeras we have shown that
oliferative response to a thymus-de-
nt antigen, ovalbumin (OVA), is
greater when the antigen is presented
ls of the parental strain used for the
marrow injection than on cells of the
parental strain.

erials and methods. Animals. BALB/
, CAF$_1$ (BALB/c × A/J), and LAF$_1$
vere purchased from Jackson Labora-
(Bar Harbor, Maine).

unization. Mice were immunized
00 μg of OVA in complete Freund's
nt (CFA) in the footpad.

Pulsing of spleen cells with antigen. The cells used were normal spleen cells which were incubated at 37° for 7 hr in a petri dish, and the nonadherent cells were transferred to a separate dish. The nonadherent cells and in one experiment the adherent cells were then incubated for 5 hr with or without 10 or 100 μg OVA ml 4 × 10^6 cells. Preliminary experiments demonstrated the requirement for 5-hr incubation with antigen to yield maximum stimulation. The cells were then washed three times to remove excess antigen. The washed spleen cells were then mixed with lymph node cells from mice immunized in the footpad 2 to 3 weeks earlier. In some cases 25 μg of mitomycin C was added to the spleen cells for the last 3 min of incubation. In one experiment the spleen cells were heated to 56° for 30 min after washing, a procedure found to produce nearly 100% loss of ability to exclude dye.

Culture conditions. Culture conditions were similar to those previously described (8). Two million immune lymph node cells plus 2 × 10^6 spleen cells (control or pulsed) were cultured at 37° in 1 ml of RPMI-1640 medium tubes in a humidified CO_2 incubator (5% CO_2 and 95% air). The medium contained 5% heat-inactivated rat serum, 100 units of penicillin G, and 100 μg of streptomycin/ml. After 3 days of culture 1 μCi of tritiated thymidine (T-^{3}H) (6 Ci/mmole) was added for the final 5 hr and DNA precipitated on glass fiber filters (Whatman GF/C grade) with trichloracetic acid (TCA) and counted in a liquid scintillation counter.

Labeling ovalbumin with ^{125}I. To 5 mCi carrier-free ^{125}I in 0.1 ml was added in sequence 0.05 ml of 0.01 N KI, 0.5 N HCl dropwise until acid, 0.05 ml of 0.1 N $NaNO_2$, 0.05 ml of 0.1 M urea, and 5 mg of OVA in 0.5 ml 0.8% NaCl solution (cf. refs. 9 and 10). The mixture was immediately brought to pH 11 by dropwise addition

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served.

of 0.2 *M* sodium carbonate buffer (pH 12) and as soon as iodine color disappeared, was brought back to neutrality by dropwise addition of 0.1 HCl. The labeled OVA was then dialyzed at 4° for 4 days against dilute KI solution with daily changes. The resulting ^{125}I-OVA contained 80,000 counts per µg (Folin) and 98% of the radioactivity was precipitable by TCA.

Preparation of chimera. CAF$_1$ mice were lethally irradiated (800 R of ^{60}Co) and reconstituted with 10 × 10^6 BALB/c bone marrow cells. A test for chimerism was made after 4 weeks on spleen cells taken from the reconstituted mice and separated twice on glass-bead columns (11). These separated spleen cells, which are mostly T-cells, were incubated with BALB/c anti A serum prepared according to the method of Miller and Mitchell (12), washed, and then mixed first with rabbit anti-mouse immunoglobulin serum and then with goat anti-rabbit immunoglobulin serum labeled with fluorescein. Twelve percent of splenic T-cells from the reconstituted mice were labeled by this technique compared to 53% of splenic T-cells from normal CAF$_1$ mice and 5% of splenic T-cells from BALB/c mice. This indicated that the majority of splenic T-cells were donor BALB/c type. The chimeras were then immunized with OVA in CFA in the footpad. Two to three weeks later, cells from their lymph nodes were removed and cultured with BALB/c or A/J spleen cells in the regular manner.

Results. Stimulatory capacity of normal spleen cells. Mice were immunized with OVA in CFA in the footpad. Two to three weeks postimmunization, single cell suspensions of the popliteal and mesenteric lymph nodes were cultured with nonadherent spleen cells previously incubated with antigen. The results of two such experiments are shown in Table I and demonstrate the excellent stimulatory capacity of the pulsed spleen cells in the mouse system. The amount of radioactivity incorporated into the lymph node cells stimulated by antigen-pulsed spleen cells (group Ic) was nearly as great as when 100 µg of antigen were left in the culture for 3 days (group Ib). In Experiment II, the OVA used for coating the normal spleen cells was labeled with ^{125}I. After three washings the amount of ^{125}I-labeled OVA that remained with the spleen cells was 0.1 µg. This amount of cell-bound OVA was almost as effective (89%) as 100 µg of free OVA in stimulating lymph node

TABLE I. THE RESPONSE OF LYMPH NODE CELLS FROM IMMUNIZED MICE TO ANTIGEN-PULSED SPLEEN CELLS TREATED WITH MITOMYCIN C AND HEAT.[a, b]

Group	Antigen in 5-hr spleen cell culture (µg)[c]	Treatment of spleen cells after 5-hr culture[d]	Antigen in 3-day mixed culture (µg)	cpm ± SE	
				Expt. 1	Expt. 2
I a	None	None	None	5,400 ± 70	5,200 ± 100
b	None	None	100	26,000 ± 1,000	25,400 ± 1,800
c	100	None	None	20,100 ± 900	23,300 ± 300
d	100	None	100	33,200 ± 2,700	27,500 ± 1,300
II a	None	Mit C	None	1,400 ± 200	1,000 ± 90
b	None	Mit C	100	12,000 ± 500	10,500 ± 450
c	100	Mit C	None	9,300 ± 900	9,300 ± 500
d	100	Mit C	100	14,600 ± 300	15,000 ± 800
III a	None	Heat	None	2,700 ± 400	–
b	None	Heat	100	15,600 ± 400	–
c	100	Heat	None	2,500 ± 80	–
d	100	Heat	100	15,700 ± 2,600	–

[a] BALB/c mice were immunized in the footpad with 400 µg of OVA in CFA and after 2 to 3 weeks popliteal lymph nodes were removed and cell suspensions prepared.

[b] Normal BALB/c spleen cells were incubated for 1 hr at 37° in Falcon plastic culture petri dishes to remove adherent cells. The nonadherent cells were cultured for 5 hr at 37° with or without OVA. The cells were then spun down and washed twice. Spleen cells 2 × 10^6 were then incubated with 2 × 10^6 immune lymph node cells for 3 days, with 1 µCi of ^{3}H-T added for the last 5 hr.

[c] In Experiment II, the OVA added to spleen cells was labeled with ^{125}I.

[d] Spleen cells were washed three times (None), treated with 25 µg of mitomycin C for the final half-hour of 5-hr culture, and washed three times (Mit C), or were washed three times and then heated at 56° for 30 min (Heat), before they were mixed with immune lymph node cells.

In other experiments the addition of of free OVA left in the 3-day culture nly 23% stimulatory as 100 μg (data iven).

ect of treating spleen cells with mitomy- or heat. The results in Table I (group ow that mitomycin C treatment of the n cells at the end of the 5-hr culture ed the net counts incorporated as a of the antigen added but increased the of experimental to control counts. the antigen-pulsed spleen cells were d at 56° for 30 min before addition of node cells, however, they completely he ability to stimulate (group IIIc). indicates that the antigen remaining the cells was insufficient to stimulate s bound to a living cell. The heated were not inhibitory to a response to n added to the 3-day culture (groups nd d).

lure of BALB/c lymph node cells to nd to antigen on allogeneic LAF$_1$ cells. Spleen cells from syngeneic B/c as well as allogeneic (LAF$_1$)] mice pulsed with antigen and mixed with B/c immune lymph node cells. The re- from these experiments given in Table w that antigen associated with LAF$_1$ cells was no more stimulatory to B/c immune lymph node cells than spleen cells alone. The incremental of adding antigen to the mixed culture learly present, however. Because of gh background of stimulation from the lymphocyte reaction we made chi- by lethally irradiating CAF$_1$ mice and reconstituting them with BALB/c bone marrow cells. The BALB/c cells which repopulated the lymph nodes of these chimeras were presumably tolerant to A/J antigens because they responded only slightly in a mixed lymphocyte reaction. Chimeras thus prepared were immunized with OVA and their lymph node cells cultured as before with OVA-pulsed spleen cells from BALB/c and A/J mice. The response to OVA of these chimeras was lower than with normal mice, but the results shown in Table III confirm the requirement that antigen be presented on syngeneic spleen cells. Antigen associated with BALB/c spleen cells was considerably more stimulatory than antigen associated with A/J spleen cells. The slight stimulation by the latter may have been due to incomplete substitution in the chimeras.

Discussion. One-tenth microgram of OVA bound to cells was almost as effective as 100 μg of free OVA in stimulating a proliferative response of lymph node cells from immunized mice. The relatively greater efficiency of bound as compared to free antigen may be much higher than these results indicate since most of the cell associated antigen may have been irrelevant (i.e., in the wrong place or on the wrong cell), and the "free" antigen probably becomes "bound" when it is added to the culture. There is now considerable reason to doubt that free antigen is recognized by T-cell directly since T-cells are not absorbed by antigen columns (13) and the specificity of the T-cell for antigen appears to always include

BLE II. FAILURE OF BALB/c LYMPH NODE CELLS TO RESPOND TO ANTIGEN ON ALLOGENEIC SPLEEN CELLS.

in source of leen cells	Antigen in 5-hr culture of spleen cells (μg)	Antigen in 3-day mixed culture (μg)	cpm ± SE Expt. 1	cpm ± SE Expt. 2
BALB/c	None	None	750 ± 60	700 ± 40
BALB/c	None	100	5,700 ± 700	5,000 ± 200
BALB/c	100	None	3,600 ± 200	3,000 ± 25
BALB/c	100	100	7,300 ± 40	7,400 ± 600
LAF$_1$	None	None	20,900 ± 1,500	28,200 ± 2,100
LAF$_1$	None	100	27,100 ± 1,000	34,300 ± 2,500
LAF$_1$	100	None	20,600 ± 1,800	21,400 ± 150
LAF$_1$	100	100	26,200 ± 1,400	32,000 ± 2,800
ponse to antigen on BALB/c cells			2,850	2,300
ponse to antigen on LAF$_1$ cells			−300	−6,800
ponse to free antigen in presence of BALB/c cells			4,900	4,300
ponse to free antigen in presence of LAF$_1$ cells			6,200	6,100

TABLE III. RESPONSE OF LYMPH NODE CELLS FROM IMMUNIZED BALB/c TO CAF$_1$ CHIMERAS TO ANTIGEN ON PARENTAL SPLEEN CELLS.[a]

Experiment	Source of spleen cells	cpm ± SE when spleen cells[b] cultured			Ratio
		Without antigen	with antigen	Net cpm	
I	BALB/c	1,200 ± 83	3,800 ± 100	2,600	3.2
	A/J	3,100 ± 80	4,900 ± 200	1,800	1.6
II	BALB/c	700 ± 26	2,600 ± 400	1,900	3.6
	A/J	2,000 ± 100	2,100 ± 20	100	1.0
III	BALB/c	500 ± 80	2,500 ± 400	2,000	5.0
	A/J	1,300 ± 0	1,700 ± 65	400	1.3
IV	BALB/c	700 ± 300	2,000 ± 100	1,300	2.9
	A/J	800 ± 47	1,000 ± 50	200	1.2

[a] Chimeras were prepared by injecting BALB/c bone marrow cells into lethally irradiated CAF$_1$ mice. Four weeks later the chimeras were immunized with OVA in the footpad. Lymph node cells from these mice were later cultured with BALB/c or A/J spleen cells preincubated with or without 100 μg of OVA.

[b] Spleen cells were prepared as in Table I, except in Expt. III where the adherent cells were used.

the H-2 antigen of the presenting cell (1-7). This may be true of non-H-2 intrisic cell surface antigens as well as of extrinsic protein antigens such as OVA (6, 7). It is difficult to understand why there should be such an inclusion of H-2 specificity if non-H-2 antigens could be recognized by T-cells directly.

The present experiments illustrate a possible method of investigating the specificity of T-cells and answering the questions discussed above. Since the only parental cells in the chimeras we produced were bone marrow-derived cells, the specificity of the responding cells for antigen presented on cells of the parental strain injected implies that under *in vivo* immunizing conditions, antigen is presented to the T-cell by a bone marrow-derived cell. An important question that may be answerable by these techniques is whether BALB/c T-cells would respond to antigen presented on A/J cells if they are first sensitized by antigen on A/J cells. We would predict that if sensitization can be accomplished by antigen-pulsed allogeneic cells, the T-cells of such an animal would respond better to antigen presented on allogeneic cells of the same strain than to antigen presented on syngeneic cells.

These experiments do not answer the question which bone marrow-derived cell or cells (B, T, or monocyte) is responsible for presenting the antigen.

It is possible as proposed by Rosenthal and Shevach (7) and Greineder and Rosenthal (14) that the macrophage is the essential cell in both antigen presentation and stimulation in mixed lymphocyte reactions. In experiments with mouse cells, small numbers of macrophages were highly stimulatory to allogeneic cells with which they were in contact for only a few hours (11, 15). Thus the nonadherent spleen cells used in these experiments may have been effective in presenting antigen because they contained only the minimum number of macrophages required.

Summary. Ovalbumin-pulsed spleen cells were found to stimulate thymidine uptake of lymph node cells of syngeneic mice immunized with ovalbumin in complete Freund's adjuvant after treatment of spleen cells with Mitomycin C but not after heating the spleen cells at 56° for 30 min. Ovalbumin-pulsed spleen cells of allogeneic mice failed to stimulate the immune lymph node cells more than unpulsed cells, although a net increase in the thymidine uptake above the allogeneic stimulation was observed when free ovalbumin was added to the mixed culture. To eliminate the high background of the mixed lymphocyte reaction, F$_1$ mice were made chimeric with bone marrow of one of the parental strains. Using lymph node cells of the immunized chimeras, the stimulation by pulsed spleen cells was much greater when antigen was presented on cells of the parental strain used for bone marrow injection than when presented on cells of the other parental strain.

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Biochemical Characteristics of Rat C-Type Virus WF-1[1] (39340)

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The discovery and morphological-immunological properties of the rat C-type virus WF-1, infectious for normal rat embryo cell cultures, have been previously described (1, 2). This presumably endogenous C-type virus was spontaneously released by a cell line (WF-1) derived from normal Wistar-Furth rat embryos. The virus was also shown to be antigenically related to two C-type viruses (RMTDV and R-35) produced by cell lines derived from rat mammary tumors (3).

Although all three viruses are similar morphologically and antigenically, only RMTDV had been found to produce some leukemogenic effects when inoculated into rats (4).

It was therefore of interest to determine the pertinent biochemical characteristics of the WF-1 virus, non-oncogenic for rats, and to compare them with those of known oncogenic RNA viruses of other animal species. The results of this study are presented in this report.

Materials and methods. Reagents. TNE buffer (0.01 *M* Tris, 0.1 *M* NaCl, and 0.001 *M* EDTA, pH 7.2), TNM buffer (0.01 *M* Tris, 0.1 *M* NaCl, and 0.001 *M* $MgCl_2$), and sucrose solutions prepared in TNE buffer were sterilized by filtration (0.45-μm porosity, Millipore Corp., Bedford, Mass.). Ribonuclease-free sucrose, [^{3}H]uridine (sp act 25 to 28 Ci/mmole), [^{14}C]uridine (sp act 36.5 mCi/mmole), and [^{3}H]uridine triphosphate (sp act 15 Ci/mmole) were purchased from Schwarz/Mann, Orangeburg, N.Y. [^{3}H]Thymidine triphosphate ([^{3}H]TTP, sp act 18 Ci/mmole) was purchased from New England Nuclear, Boston, Mass. Electrophoretically purified nucleases (RNase, code Rase; and DNase, code DPFF) were purchased from Worthington Biochemical Corp., Freehold, N.J.

Other reagents were: 2-mercaptoethanol (2-ME), dithiothreitol (DTT), dATP, dCTP, dGTP, rATP, rCTP, rGTP (Sigma Chemical Co., St. Louis, Mo.), Nonidet P-40′ (Shell Chemical Co., New York, N.Y.), diethylpyrocarbonate (DEP), diethyl oxydiformate (Eastman Kodak Co., Rochester, N.Y.), and trichloroacetic acid (TCA).

Cell cultures and meda. The origin and propagation techniques of WF-1 cells have been described (1). For the studies reported here, WF-1 cells between the sixteenth and twenty-eighth *in vitro* passages were used. The growth medium was Eagle's minimal essential medium containing 100 units of penicillin G and 100 μg of streptomycin per milliliter and 10% fetal calf serum (MEM-FCS). Rat embryo (RE-2) cells, grown in MEM-FCS, were obtained from Dr. N. Biswal, Baylor College of Medicine. Chicken embryo fibroblasts (SPAFAS, CEF) fully transformed by Rous Sarcoma Virus (Rous-Associated-Virus) [RSV(RAV-1)] were kindly supplied by Dr. P. Meyers.

Virus purification. Culture supernatants were harvested at 10- to 14-hr intervals after the addition of 10 μCi/ml of [^{3}H]uridine to the WF-1 cells or 20 μCi/ml of [^{3}H]uridine to the transformed CEF cells. The fluids were clarified at 3000 *g* for 10 min and concentrated to approx 10 ml by diaflo ultrafiltration at 4° under positive nitrogen pressure. The 402 diaflo unit (Amicon Corp., Lexington, Mass.) was autoclaved before use, and the centrifuge tubes

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he Amicon PM-30 membrane were zed with uv light. Concentrates were ied at 10,000 *g* for 10 min.

ncentrates were layered onto discon- us gradients of 8 ml of 20% sucrose oned by 5 ml of 55% sucrose and then ifuged at 25,000 rpm for 2 hr in a o SW 25.1 rotor at 4°. The band at the phase was collected by puncturing the m of the tube. It was diluted with TNE r, and layered over a 20-ml linear gra- of 20 to 55% sucrose. After 8 to 10 hr ntrifugation as described above, frac- of approx 1 ml were collected from the m of the tube. The radioactivity in a l sample of each fraction was measured ml of Beckman Ready-Solv VI (Beck- Instruments, Inc., Fullerton, Calif.) or acid-insoluble counts retained by Mil- filters (5) in 10 ml of Beckman y-Solv IV in a Packard Tri-Carb Model scintillation spectrometer at 4°. The nt density of each fraction was deter- d by refractometry.

A extraction. Appropriate gradient ons were combined and then diluted TNE buffer to contain less than 10% se. To each 10-ml vol were added 0.1 2-ME, 1 ml of 1% DTT in 1% phenol, .2 ml of DEP. After mixing in an ice for 10 min, the preparations were ed to approx 20°, and SDS was added e a final concentration of 1%. Prepa- ns were deproteinized with PMQN ml of freshly distilled phenol saturated TNE buffer, 12 ml of freshly distilled sol, and 0.1 g of 8-hydroxyquinoline, .4) as described by Biswal and Ben- Melnick (5). The RNA in the aqueous was precipitated at −20° for at least 2 er adding one-tenth vol of 20% Na e (pH 5.2) and 2 vol of iced 95% ol. The RNA precipitate was collected ntrifugation at 15,000 *g* for 20 min and issolved in TNE buffer.

ernatively, radioactive virions from terphase of the discontinuous gradient m the appropriate gradient fractions pelleted at 25,000 rpm for 1 hr in the 5.1 rotor. The pellet was resuspended ml of TNE buffer containing 2-ME, and DEP (as described), and the was extracted with 1% sodium dode- fate (SDS).

Centrifugation of RNA. RNA preparations were layered onto 4.6-ml linear gradients of 5 to 20% sucrose and sedimented at 35,000 rpm for 2 hr at 4° in an SW 39 rotor. Fifteen-drop fractions were collected from the bottom of the tube into tubes containing 0.4 ml of TNE buffer. The radioactivity in each fraction was assayed as the acid-precipitable material (5) in 10 ml of Beckman Ready-Solv IV. Sedimentation coefficients were determined by the method of Martin and Ames (6) using ^{14}C-labeled 28 S ribosomal RNA (rRNA) isolated from RE-2 cells or ^{3}H-labeled high mol wt RNA isolated from RSV(RAV-1).

RNA was recovered from gradients by collecting 15-drop fractions into tubes containing 1 ml of TNE buffer, 50 μg of unlabeled rRNA, and 0.1% (v/v) DEP. The RNA in selected fractions was precipitated with Na acetate and ethanol as described above.

Reverse transcriptase assay. Culture supernatants were harvested, concentrated, and purified as described herein. The virus concentration was based on the amount of protein present in the viral band as determined by the method of Lowry *et al.* (7). The endogenous polymerase assay was modified from the method of Spiegelman *et al.* (8). A standard incubation mixture of 0.1 ml contained: 0.2 mmole each dATP, dCTP, and dGTP; 0.109 μmole [^{3}H]TTP (15–18 Ci/mmole); 2.8 mmole 2-ME; 12 mmole MgCl$_2$; 40 mmole KCl; and 57 mmole Tris–HCl (pH 8.3). Virus suspended in Tris–HCl (pH 8.3) was preincubated for 10 min at 0° with 0.08% Nonidet P-40 and 0.4% 2-ME. The preincubation mixture was added to the incubation mixture and placed immediately at 37°. After incubation for 180 min, each sample was co-precipitated with 50 μg of bovine serum albumin by the addition of 0.3 ml of TCA mixture (equal volumes of 100% TCA, saturated NaH$_2$PO$_4$, and saturated Na$_4$P$_2$O$_7$·10 H$_2$O). After 10 min, the precipitate was collected on nitrocellulose filters and washed with 5% TCA and then with 2 ml of 95% ethanol. The radioactivity on dried filters was determined as described herein.

RNA-dependent RNA polymerase (RDRP) assay. The incubation mixture consisted of 0.25 ml containing (in micromoles): 1.0 each of rATP, rCTP, and rGTP; 2.67×10^{-4} of ^{3}H-rUTP; 3 of $MgCl_2$; 10 of KCl; and 14.25 of Tris-HCl (pH 8.3). The preparation of virus, preincubation mixture, and assay for radioactivity were done as described above.

Results. Cultures of WF-1 cells were labeled with [^{3}H]uridine, and the supernatant fluids were tested for the presence of radioactive virus particles by equilibrium centrifugation in sucrose gradients. The RE-2 cells, in which C-type particles have not been seen in numerous examinations by electron microscopy, were treated in a similar manner. Figure 1 demonstrates that culture fluids from WF-1 cells produced a prominent peak of radioactivity of a buoyant density of 1.15 to 1.16 g/cm^3. Culture fluids from RE-2 cells labeled with [^{3}H]uridine, or from WF-1 cells labeled with [^{3}H]thymidine, failed to yield a similar peak.

Volumes of 300 to 450 ml of freshly harvested fluids from radiolabeled WF-1 cell cultures were concentrated, purified, and used for isolation of viral RNA. The RNA from purified virions was extracted with SDS-PMQN and sedimented in 5 to 20% linear sucrose gradients. Approximately 55 to 65% of the RNA was located in the 60 to 70 S region of the gradient. In some experiments, radiolabeled virions were collected from the interphase of discontinuous gradients and RNA was extracted with 1% SDS. There were no differences in sedimentation coefficients of RNA species obtained by either of these methods. When compared to the position of 28 S rRNA in several gradients, WF-1 RNA sedimented at 62 to 68 S (Fig. 2). Sedimentation of RNA from RSV(RAV-1) and WF-1 virions, either in separate gradients or in the same gradient, indicated that the RNA from RSV(RAV-1) sedimented slightly faster than the RNA from WF-1 virus (Fig. 3). Accepting a sedimentation coefficient of 71 S for the avian virus RNA (9), WF-1 viral RNA sedimented at approx 64 S. It has also been reported that high mol wt RNA from RSV(RAV) sediments slightly faster than the high mol wt RNA from mouse mammary tumor virus (9, 10).

The experiments described above were done with RNA extracted from virions harvested after 10- to 14-hr incubation. However, when WF-1 cells were labeled for 24 to 48 hr, or when multiple harvests were made and each harvest was held at 4 or −20° until the final harvest, the recovery of high mol wt RNA ranged from 0 to 40%, with the remainder of the cpm spread over a range of 4 to 40 S. Virus preparations that were frozen and thawed only once yielded primarily (80 to 100%) 4 to 12 S RNA. In

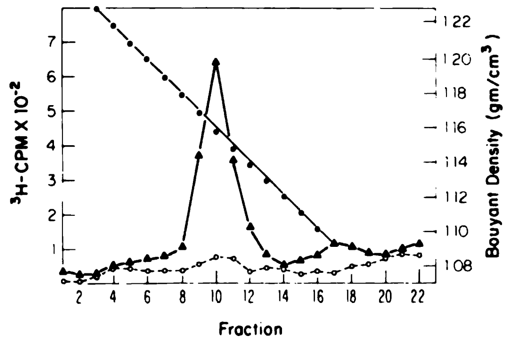


FIG. 1. Density gradient centrifugation of culture fluids from WF-1 cells. Cultures were labeled for 12 hr with 10 µCi/ml of [^{3}H]uridine (▲——▲) or [^{3}H]thymidine (O- - -O). Supernatant fluids were concentrated and purified by centrifugation in sucrose gradients. Fractions of approx 1 ml were collected from the bottom of the tube, and a 50-µl sample of each fraction was assayed for acid-precipitable radioactivity.

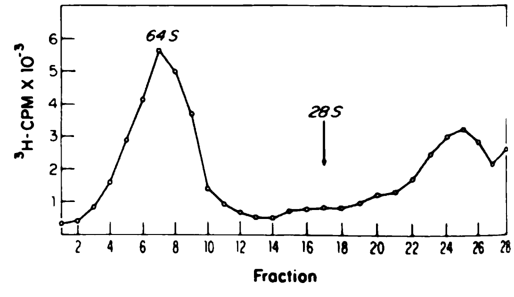
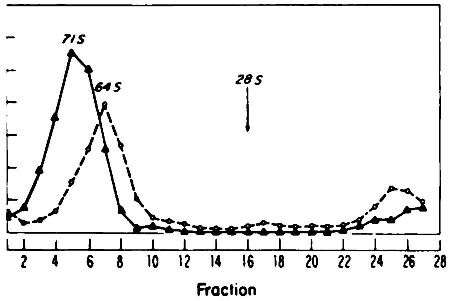


FIG. 2. Velocity sedimentation of viral RNA. Radioactive WF-1 virions were collected from the interphase of a discontinuous 20 to 55% sucrose gradient, pelleted, and lysed as described in the text. The ^{3}H-labeled viral RNA was centrifuged with ^{14}C-labeled 28 S rRNA in a 5 to 20% sucrose gradient at 35,000 rpm for 2 hr in a Spinco SW 39 rotor. Fractions of 15 drops were collected from the bottom of the tube and assayed for acid-precipitable radioactivity. The arrow in this and the following figures shows the position of the peak of ^{14}C-labeled 28 S rRNA from normal rat embryo cells.



. 3. Velocity sedimentation of RNA. Depro- d preparations of ^{3}H-labeled RSV(RAV-1) (▲——▲) and WF-1 RNA (O- - -O) were sedi- d in separate 5 to 20% sucrose gradients. The NAs had been separated from the more slowly nting RNA components by preparative sucrose nt centrifugation (see Methods). The procedures ntrifugation, gradient fractionation, and assay for ctivity are as described in Fig. 2.

of these preparations, as much as 1 mg labeled 28 S rRNA or 2000 cpm of beled 28 S rRNA was added prior to ption of the virus. Assays of gradient ons for ^{14}C-radioactivity or optical den- ndicated good recovery of 28 S rRNA, sting that the RNA was not degraded g the disruption and extraction proce- ;.

ese results suggest that long labeling vals and storage at 4 or −20° may cause dation of the 60 to 70 S viral RNA components which sediment more y. Similar results have been reported in es of RNA extracted from other oncor- uses (9, 11, 12).

e RNA precipitated from the 60 to 70 ion of the gradient was dissolved in 0.2 aCl for alkaline hydrolysis or in TNM r for assay with RNAse. Duplicate les containing 1850 cpm of acid-pre- ible radioactivity retained an average cpm (1.4%) of acid-insoluble material treatment with 25 μg of RNase at 37° 0 min. After treatment with an equal ne of 1 *M* NaOH at 80° for 30 min, cate samples containing 3674 cpm each d only 0.4% of acid-insoluble radioac- . These results indicate that the high vt RNA is single-stranded and that no than 0.4% of the nucleic acid could been DNA.

atment of the high mol wt RNA of rous oncogenic RNA viruses with heat or DMSO converts the 60 to 70 S RNA into more slowly sedimenting components, indicating that the high mol wt RNA consists of subunits. Figure 4 depicts the results obtained upon centrifugation of WF-1 high mol wt RNA after the sample was heated at 100° for 2 min in a sealed ampule. In comparison with unlabeled 28 S rRNA, the heated viral RNA dissociated into components of approx 36 S, 18 to 20 S, and 4 to 12 S. Similar results have been reported for heat-dissociated high mol wt RNA from murine sarcoma-leukemia virus (13).

In reverse transcriptase assays, the incorporation of [^{3}H]TTP into acid-insoluble material by the virion-associated reverse transcriptase was decreased by the omission of $MgCl_2$ or two of the deoxynucleotide triphosphates (Table I). The reaction was only slightly affected by the absence of 2-ME, but disruption of the virions was necessary for maximum polymerase activity. If the disrupted particles were treated with RNase before addition to the incubation mixture, a 47% loss of precipitable counts resulted, indicating dependence upon the presence of RNA for maximum activity. The product of polymerization using this endogenous template was not affected by RNase but was rendered acid-soluble by DNase treatment (Table II), indicating the presence of DNA in the product. However, annealing experi-

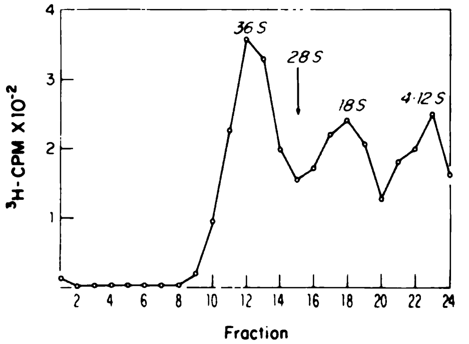


FIG. 4. Velocity sedimentation of heat-dissociated WF-1 viral RNA. ^{3}H-labeled viral RNA was separated from more slowly sedimenting RNA components by preparative sucrose gradient centrifugation and heated at 100° for 2 min in a sealed ampoule. Centrifugation was in a linear 5 to 20% sucrose gradient at 35,000 rpm for 2.5 hr in a Spinco SW 39 rotor. Gradient fractionation and assay for radioactivity were performed as described in Fig. 2.

TABLE I. REQUIREMENTS OF WF-1 VIRUS-ASSOCIATED REVERSE TRANSCRIPTASE.

Condition[a]	^{3}H [TTP] incorporated[b] (cpm)
Complete assay system	1175
Complete, RNase preincubation[c]	627
No virus	276
No dCTP and dGTP	352
No $MgCl_2$	432
No 2-ME	957
No Nonidet P-40	512

[a] Assay conditions using the endogenous template are described in Methods. The preincubation mixture contained 80 μg of protein.

[b] Total cpm in 0.1-ml test volumes, as the average of two tests.

[c] To the preincubation mixture after the initial 10 min of incubation was added 10 μl of RNase (1 mg/ml) and 1.2 μmole $MgCl_2$, which usually was in the standard incubation mixture. The preincubation mixture plus RNase was incubated at 37° for 15 min before addition to the standard incubation mixture.

TABLE II. PROPERTIES OF THE PRODUCT OF VIRION-ASSOCIATED REVERSE TRANSCRIPTASE.[a]

Treatment[b]	cpm[c]	Percentage cpm recovered[d]
None	1462	100
RNase (100 μg/ml)	1551	106.1
DNase (100 μg/ml)	548	37.3

[a] Assay conditions using the endogenous template are described in Methods. The preincubation mixtures contained 80 μg of protein.

[b] After reaction at 37° for 3 hr, 0.1-ml aliquots were treated as follows: No treatment but further incubation at 37° for 90 min; RNase treatment for 90 min at 37°; DNase treatment for 90 min at 37°.

[c] Total cpm in 0.1-ml test volumes, as the average of two tests.

[d] Untreated sample assumed as 100% reaction.

ments to demonstrate complementarity of the product to viral high mol wt RNA were not done.

Assays to detect possible RDRP were also performed. All attempts to detect this enzyme in association with WF-1 virus were unsuccessful.

Discussion. The results of the present study indicate that the virus isolated from WF-1 cells has several biochemical characteristics in common with known oncogenic C-type viruses.

The buoyant density of the WF-1 virus in sucrose gradients is 1.15 to 1.16 g/cm^3. This density is slightly greater than that (1.14 g/cm^3) previously reported for WF-1 virus (2). This may reflect the differences in the time intervals used for labeling the virus (10 to 14 hr as opposed to 48 hr) and the methods employed to process supernatant culture fluids and purify the virus. The C-type RNA tumor viruses contain single-stranded 60 to 70 S RNA which, upon treatment with heat or DMSO, dissociates into components which sediment more slowly, suggesting a subunit structure. As demonstrated here, the WF-1 virus possesses single-stranded RNA with a sedimentation coefficient (approx 64 S) slightly less than that of the RNA from RSV(RAV-1) (71 S). The results obtained upon sedimentation of heat-dissociated high mol wt RNA suggest that the WF-1 viral RNA may be composed of subunits.

The presence of the WF-1 virus reverse transcriptase is indicated by the *in vitro* synthesis of a DNA-like product when disrupted virions are present in the assay. Preincubation of disrupted virus preparations with RNase significantly decreased the incorporation of [^{3}H]TTP, indicating that the template is RNA, and the product of the polymerase assay was sensitive to DNase but not to RNase. The enzyme thus shares some of the characteristics attributed to polymerases from various other C-type viruses (14–16). No RDRP activity could be detected in association with the virus.

To date all attempts to demonstrate a biological activity of the WF-1 virus in rats have been unsuccessful despite of: (a) its ability to infect rat cells *in vitro* (2), and (b) high malignancy of the rat embryo cell line (1) from which the WF-1 virus was isolated. It appears at the present time that the WF-1 virus is a non-oncogenic endogenous virus of rats. However, it may be equally likely that the oncogenicity of this virus is repressed upon inoculation by host-associated factors, a possibility to be carefully considered in future studies with this and other rat C-type viruses in their natural host.

Summary. The non-oncogenic rat C-type virus WF-1, isolated from a Wistar-Furth rat embryo cell line, was characterized biochemically. The purified virus has a buoyant density of 1.15 to 1.16 g/cm^3 in sucrose, RNA-dependent DNA polymerase (reverse transcriptase) activity, and RNA with a sed-

imentation coefficient of 62 to 68 S. The viral RNA is single-stranded, and, upon treatment with heat, yields components with sedimentation coefficients of 36 S, 18 to 20 S, and 4 to 12 S.

We thank Ms. H. Asmussen for technical assistance, and Ms. J. G. Bronson for preparing the figures.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 122-125 (1976)

Decreased Venous Compliance in Dogs with Chronic Renal Hypertension[1] (39341)

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In essential hypertensive patients, evidence for decreased digital and forearm venous compliance was found by plethysmography (1, 2). In spontaneously hypertensive rats, Greenberg and Bohr (3) reported decreased passive extensibility of portal vein strips. In earlier studies, we found decreased compliance of femoral (4) and mesenteric veins (5) in dogs with early one-kidney perinephritic hypertension. The purpose of the present study was to extend our previous investigations by determining whether the decreases in venous compliance persist into the chronic (more than 4 weeks) stage of one-kidney perinephritic hypertension in dogs. We observed pressure-volume relationships in isolated segments of femoral veins. We also examined the veins histologically, and measured their water and electrolyte contents in an attempt to define the mechanisms responsible for the decreases in venous compliance in hypertension.

Materials and methods. Twenty-seven healthy, male mongrel dogs, weighing 19-29 kg, were trained to lie quietly during femoral arterial punctures for weekly blood pressure measurements. Mean arterial blood pressure less than 130 mm Hg was documented in each dog prior to surgery. During the entire study period the dogs were maintained on standard dog chow (Wayne Dog Food) and water *ad libitum*. In fourteen dogs, randomly selected, one kidney was wrapped in silk and the other kidney was removed 1 week later (experimental group). Thirteen other dogs underwent sham-wrapping and contralateral nephrectomy at similar time intervals (control group). Operative procedures were similar to those we have previously described (5). Four to 24 weeks following nephrectomy, the dogs, fasted 24 hr, were anesthetized with sodium thiamylal (13.2 mg/kg iv) and mechanically ventilated. Blood was obtained for hematocrit, serum creatinine (autoanalyzer), and serum sodium and potassium (flame photometer) measurements. A 5-cm segment of femoral vein (FV), downstream from the branching of the caudal FV, was dissected *in situ*. With collaterals tied off and cut near their origins, the FV segment was removed and transferred to a chamber containing Krebs-Ringer (K-R) bicarbonate solution (NaCl, 118.3 m*M*; KCl, 4.7 m*M*; $MgSO_4$, 1.2 m*M*; KH_2PO_4, 1.2 m*M*; $CaCl_2$, 2.5 m*M*; $NaHCO_3$, 25.0 m*M*; and glucose, 11.1 m*M*) kept at 37° and aerated with 95% O_2 and 5% CO_2 gas mixture (Fig. 1). The pH of the solution was adjusted to 7.40-7.43 with 0.1 *N* HCl. After tying each end of the cut venous segment to a thin-walled glass cannula, the *in vitro* length of the vein was adjusted to 4 cm. The vein was perfused at 25 ml/min with the same K-R solution used in the chamber. To measure venous pressure-volume relationships, perfusion was stopped, the inflow and outflow tubes were clamped, and intraluminal pressure was adjusted to atmospheric pressure. The system was checked for fluid leakage by injecting K-R solution until an intraluminal pressure of 40-50 mm Hg was reached and then by retrieving all injected solution. Thereafter, FV pressure-volume relationships were obtained by rapid continuous infusion at 3.88 ml/min (Harvard infusion-withdrawal pump), reaching 40-50 mm Hg intraluminal pressure maximum in 5-15 sec. These measurements were repeated at least twice at 25-min intervals. At the end of the experiment, the venous segment was weighed to within ±0.001 g.

Using the average of two or three pressure-volume measurements, a venous pressure-volume curve was constructed for each dog. Volumes (milliliters) producing intra-

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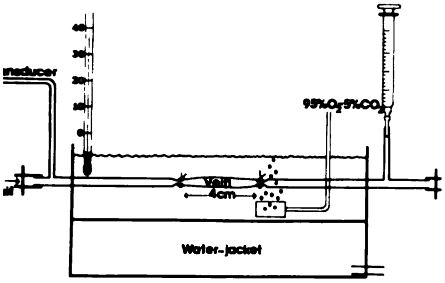

1. Schematic drawing of apparatus used to 'enous pressure-volume relationships *in vitro*.

al pressures of 5, 15, 25, and 35 mm hypertensive dogs were compared to ıponding values in normotensive con- ogs by Student's *t* test. Similarly, pres- (mm Hg) reached after the infusion of 0.26, 0.39, and 0.52 ml of fluid and ated compliances ($\Delta V/\Delta P$) in the pres- ange of 0–15 mm Hg in hypertensive were compared to corresponding val- normotensive control dogs. The data also analyzed by profile analysis (6). atter analysis takes into consideration ct that the data points were not inde- nt, thereby inducing correlation of er- In this latter analysis, we tested the ıypotheses that the pressure-volume ; of hypertensives and normotensives, he volume range of 0–0.52 ml, were el and not displaced from one another. ıese analyses, several pressures in the r volume ranges were derived by ex- ation from the individual pressure- ıe curves. Null hypotheses were re- l at $P \leq 0.05$.

ing the dissection of the FV, an addi- segment of the vein was removed for ıis of water, sodium, and potassium ıts. Water content was measured by ing before and after oven-drying the ıt 100° for 24 hr. The sodium and ıium contents were determined with a nan Model 105 Flame Photometer, ing digestion of venous tissue with 0.1 O_3 for 2 weeks. The water content, ters per kilogram wet weight, and so- and potassium contents, milliequiva- ıer kilogram dry weight, of veins from ensive dogs were compared with cor- ıding values of control dogs by Stu- *t* test, rejecting null hypotheses at *P* 5.

Finally, in three hypertensive and three control dogs, a segment of the contralateral FV was removed for histological examination. Serial sections of veins were stained with hematoxylin and eosin, Wilder's reticulin, Verhoff's elastica, Gomori's trichome, Lillie's allochrome, and AB PAS stains.

Results. Initially, mean arterial blood pressure (MABP) in the experimental group was 113 mm Hg ($N = 14$). After one kidney was wrapped in silk and the opposite kidney removed, MABP in unanesthetized dogs rose to 170 mm Hg ($P < 0.001, N = 14$) at the time of the venous pressure-volume studies. The average time from the induction of perinephritis to the time of the pressure-volume studies was 66 days ($N = 14$). The average duration of hypertension (statistically significant increase in MABP) was 44 days ($N = 13$). Hypertension was present in all dogs for at least 4 weeks. Initially, MABP in the control group was 109 mm Hg, and there was no significant change ($P > 0.5, N = 13$) in MABP following sham surgery and contralateral nephrectomy. At the time of the hemodynamic studies, the mean body weights of hypertensive and of control dogs, 25.8 and 25.5 kg, were not significantly different ($P > 0.5$). The general health of all dogs remained good. The venous blood hematocrit (volume percent) of hypertensive and normotensive dogs at the time of the hemodynamic studies was within normal limits. The serum creatinine and serum concentrations of sodium and potassium in hypertensive ($N = 7$) and control normotensive ($N = 7$) animals were also within normal limits and not significantly different ($P > 0.5$). The mean weight of the femoral vein (FV) segments from hypertensive dogs used for pressure-volume studies (0.125 g) did not significantly differ ($P > 0.1$) from that of control dogs (0.137 g).

In hypertensive dogs, the volumes infused to reach 15, 25, and 35 mm Hg were significantly less ($P < 0.05$) (Fig. 2) and the intraluminal pressures reached after the infusion of 0.39 and 0.52 ml of fluid were significantly greater ($P < 0.05$) (Table I) than in normotensive dogs. In the pressure range of 0–15 mm Hg, the calculated femoral vein compliance of hypertensive dogs was significantly reduced compared to that of normotensive dogs ($P < 0.05$) (Table I). The pressure–volume curves of both hypertensive and normotensive dogs were convex toward the volume axis. There was a statistically significant shift of the pressure–volume curves of hypertensive dogs toward the pressure axis (Fig. 2). This displacement of the pressure–volume curve in hypertensive dogs was confirmed by profile analysis ($P < 0.05$); this analysis, however, indicated that the two curves were parallel ($P > 0.2$), i.e., their shapes were similar.

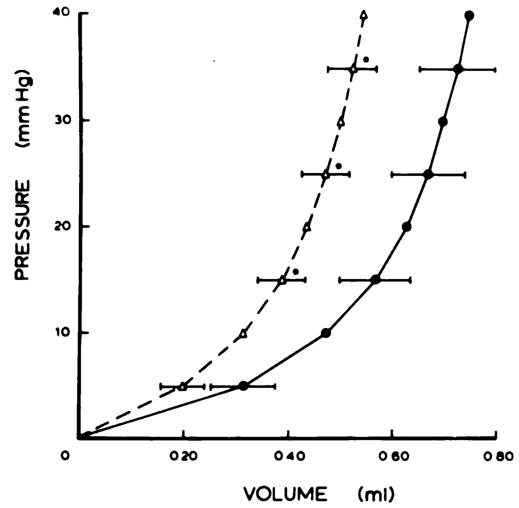

FIG. 2. *In vitro* femoral vein pressure–volume curves, means of groups. Solid and interrupted lines indicate the pressure–volume curve of control ($N = 13$) and hypertensive ($N = 14$) dogs, respectively. Horizontal bars represent ±SEM; * $P < 0.05$.

TABLE I. *IN VITRO* FEMORAL VEIN PRESSURE–VOLUME RELATIONSHIPS AND COMPLIANCES.[a]

	Control ($N = 13$)	Hypertensive ($N = 14$)
	Pressure (mm Hg)	
Volume (ml)		
0.13	3.3 ± 0.6	5.2 ± 0.9
0.26	6.5 ± 1.3	13.0 ± 2.9
0.39	11.0 ± 2.9	27.0 ± 6.8*
0.52	20.0 ± 5.1	46.0 ± 10.4*
	Compliance, $\Delta V/\Delta P$ (ml/mm Hg)	
Pressure range (mm Hg)		
0–15	0.0376 ± 0.0045	0.0256 ± 0.0031*

[a] Results are expressed as group means ± SE.

* $P < 0.05$, for comparisons between hypertensive and control groups.

The water and sodium contents of the femoral veins from hypertensive dogs were significantly greater than those of veins from control dogs (Table II).

Histological examination of the stained sections of the femoral veins provided no evidence for venous wall abnormalities in hypertensive dogs. Specifically, there was no evidence for smooth muscle hypertrophy or proliferation, increased adventitial collagen, reticulin or fibrous fibers, accumulation of neutral or acid mucopolysaccharides, and endothelial cell hyperplasia and/or swelling.

Discussion. In a previous study, Overbeck (4) observed that the pressure–volume curve of the femoral vein (FV) of dogs with early (less than 4 weeks) one-kidney, perinephritic hypertension is shifted in the direction of the pressure axis, direct evidence that venous compliance may be decreased. In a subsequent study (5), we found that the mesenteric vein pressure–volume curve in early one-kidney perinephritic hypertensive dogs is also shifted toward the pressure axis. This was true both in *in vivo* and in *in vitro* studies. Active venoconstriction did not seem to be responsible for this decreased venous compliance because the pressure–volume curves in both hypertensive and normotensive dogs were convex toward the volume axis; venoconstriction characteristically changes the pressure–volume curve to a sigmoid configuration (7). Furthermore, in hypertensive dogs sodium cyanide, which abolishes smooth muscle tone, did not change the shape of the pressure–volume

TABLE II. VENOUS WALL WATER, SODIUM, AND POTASSIUM CONTENT IN CONTROL AND HYPERTENSIVE DOGS.[a]

	Control	Hypertensive
Water (ml/kg wet weight)	633 ± 17 $N = 10$	684 ± 11* $N = 10$
Sodium (mEq/kg dry weight)	277.6 ± 21.6 $N = 8$	353.1 ± 21.7* $N = 8$
Potassium (mEq/kg dry weight)	68.7 ± 12.7 $N = 9$	75.6 ± 24.6 $N = 9$

[a] Results are expressed as group means ± SE.

* $P < 0.05$, for comparison between hypertensive and control group.

; or prevent the shift toward the pres-
xis. Subsequently, Pamnani and Over-
(8) reported increased water, sodium,
otassium contents of veins in one-kid-
oldblatt hypertensive rats, an observa-
hat might account for the changes that
d observed in venous compliance.

: findings of our present study extend
previous observations to dogs with
ic perinephritic hypertension. Again,
se chronic renal hypertensive dogs, the
ressure-volume curves are shifted to-
the pressure axis, suggesting decreased
is compliance. Again, the convexity of
ressure-volume curves is towards the
ie axis, suggesting that the mechanism
nsible for the decreased venous com-
e does not involve active venocon-
on. By light microscopy we could not
nstrate histological changes, but we
nd increased water and sodium con-
of these same veins. Therefore, we
st that venous wall "edema" may ac-
for the decreases in venous compli-
It has similarly been suggested that
ecreased arterial compliance in dogs
enal hypertension may be attributable
reased water content of arterial walls

;arding the pathogenesis of decreased
iance, our *in vitro* studies effectively
iate the possibility that humoral and
l factors were immediately responsible
ie observed changes. However, it is
le that the decreased compliance and
normal composition of the FV could
een the residual effects of humoral or
l factors.

rario *et al.* (10) found increased car-
utput in early one-kidney perinephri-
ertensive dogs. In the chronic stage of
tension, cardiac output returned to
l. Their dogs were prepared similarly
s. The decreased femoral and mesen-
ein compliance that we found in early
tension may contribute to the initial
f cardiac output by increasing venous
. However, the present study indi-
that this reduction of femoral vein
iance apparently persists into the
c stage of hypertension. If veins
hout the body are similarly affected,
he present findings suggest that the return to normal of cardiac output in the chronic stage of hypertension is due to mechanisms other than the return of venous compliance to normal.

Summary. Femoral vein (FV) pressure-volume relationships were measured *in vitro* in 14 dogs with chronic (more than 4 weeks), one-kidney perinephritic hypertension and in 13 unilaterally nephrectomized normotensive control dogs. Segments of FV were also examined histologically and analyzed for their water and electrolyte contents. Compared to controls: (i) the FV pressure-volume curves of hypertensive dogs were shifted toward the pressure axis ($P < 0.05$); (ii) calculated venous compliance in the pressure range of 0-15 mm Hg was decreased ($P < 0.05$); and (iii) the water and sodium contents of veins from hypertensive dogs were increased ($P < 0.05$). Histological examination of the FV from hypertensive and control dogs did not reveal significant differences. The findings indicate that the decreases in venous compliance that we have previously observed in the early stages (less than 4 weeks) of perinephritic hypertension in dogs persist into the chronic stage of hypertension. Venous wall "edema" may account for the decreased venous compliance in this form of hypertension.

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Inhibition of Cholera Toxin-Stimulated Intestinal Epithelial Cell Adenylate Cyclase by Adenosine Analogs[1] (39342)

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(Introduced by R. W. Wannemacher, Jr.)

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Cholera toxin elaborated by *Vibrio cholerae* binds essentially irreversibly to its membranous receptor within minutes of contact with intestinal cells (1). The resulting increase in intestinal fluid and electrolyte secretion is mediated by toxin activation of the intestinal adenylate cyclase–cyclic AMP system (2). Cholera toxin is also able to activate adenylate cyclases in a variety of nonintestinal mammalian tissues (3-5). Effective therapy for cholera now consists of replacement of fluid and electrolytes and administration of antibiotics (6). Although this therapy is very successful when initiated early, logistics is a major problem in treating patients in underdeveloped areas. Therefore, a simpler method of treating cholera is needed. Theoretically, agents which lower intestinal cyclic AMP content could either complement or, perhaps, replace current therapy.

Although various attempts have been made to blunt the cholera toxin response, little success has been achieved. Antibodies to cholera toxin which are effective in removing free toxin from solution cannot remove the toxin from its membranous receptor (7). Similarily, ganglioside G_{M1}, the toxin's membranous receptor, is effective in binding free but not bound toxin (8). A natural occuring toxoid is capable of inhibiting cholera toxin binding and thereby toxin action (9). However, since the toxoid acts by forming an inactive complex with the membrane receptor, it must be administered prior to the toxin making it unsuitable for therapeutic use. Similarily, ethacrynic acid (10) and cycloheximide (11) have been shown to reverse some of the effects of cholera toxin, but neither is suitable as a therapeutic agent for use in humans. These results suggest that other pharmacological agents might be found which would reverse cholera toxin effects and be safe for human use. In this regard, recent studies have shown that adenosine and related analogs are capable of inhibiting adenylate cyclase in a variety of systems (12-15). Therefore, the ability of these agents to inhibit cholera toxin activation of human embryonic intestinal epithelial cell adenylate cyclase was investigated.

Materials and methods. [α-^{32}P]ATP, [^{3}H]cyclic AMP, and Riafluor were purchased from New England Nuclear, Boston, Mass. Cholera toxin was purchased from Schwarz/Mann. 2′,5′-Dideoxyadenosine and 2′-fluoroadenosine were kindly supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. All other chemicals were purchased from standard sources in the highest available grade.

Tissue culture preparation. Human embryonic intestinal epithelial cells, CCL-6, were obtained from the American Type Culture Collection, Rockville, Md. Cell monolayers were grown to confluency in sterile 60- or 150-mm petri dishes. Growth media consisted of Hanks basal minimal essential medium supplemented with 15% fetal calf serum (16).

Preparation and assay of CCL-6 cell adenylate cyclase. After incubating cells in 150-mm petri dishes for 18 hr at 37° with 0.7 μg/ml of cholera toxin, monolayers were washed twice with 10 ml of RPMI-1640. Three dishes of cells were then suspended

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[1] In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council. The facilities are fully accredited by the American Association of Accreditation of Laboratory Animal Care.

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TABLE I. EFFECT OF VARIOUS ADENOSINE ANALOGS ON CHOLERA TOXIN-MEDIATED INCREASES IN CCL-6 CELL CYCLIC AMP CONTENT.[a]

Additions	Concn. (μM)	Picomoles cyclic AMP/ plate ± SEM
Experiment 1		
None		104 ± 6
2′,5′-Dideoxyadenosine	130	46 ± 4
	70	71 ± 4
	35	97 ± 5
Adenosine	130	116 ± 9
2′-Deoxyadenosine	130	83 ± 3
2-Chloroadenosine	130	121 ± 4
2-Fluoroadenosine	130	114 ± 9
7-Deazaadenosine	130	97 ± 8
Experiment 2		
None		142 ± 8
2′,5′-Dideoxyadenosine	130	78 ± 7
Adenosine 5′-sulfate	130	135 ± 3
3′-Deoxyadenosine	130	129 ± 12
Dimethylallyladenosine	130	155 ± 6
1-Methyladenosine	130	149 ± 10

[a] Cholera toxin (100 ng/ml) was added at zero time to CCL-6 cells. Test agents were present during the last 30 min, and 1-methyl-3-isobutylxanthine during the last 15 min of a 6-hr incubation. Controls contained 19 ± 2 and 12 ± 2 pmole of cyclic AMP per plate for Experiments 1 and 2, respectively. $N = 4$.

echanical scraping in a total of 20 ml of
M Tris-HCl (pH 7.6), containing 2.0
MgSO$_4$, 0.5 m*M* EDTA, and 1.0 m*M*
threitol. After homogenizing, the sus-
on was diluted to 30 ml, passed
gh gauze, and centrifuged at 3000 *g*
0 min. This pellet was homogenized by
in 1.5 ml of buffer. Twenty-five micro-
of homogenate (150–200 μg protein)
assayed for adenylate cyclase activity.
lenylate cyclase activity was measured
he conversion of [α-^{32}P]ATP into
yclic AMP as previously described us-
e alumina column method (17). Under
assay conditions, both stimulated and
imulated cyclase activities were linear
g a 10-min incubation at 30°. Protein
ntrations were estimated by the Lowry
od (18) using bovine serum albumin as
dard.

termination of cyclic AMP. Cells were
ed twice with 2 ml of RPMI-1640 to
ve serum and incubated at 37° with 2
RPMI-1640 containing 100 ng/ml of
ra toxin in 60-mm petri dishes for 6 hr.
ninary studies showed no major differ-
between 6-hr compared to 18-hr incu-
ns. After exposure to cholera toxin for
r, test agents were added. At 5.75 hr, 1
f RPMI-1640 containing 3 m*M* 1-
yl-3-isobutylxanthine (MIX) was
d. The incubation was terminated at
nd of 6 hr by removing the media and
g 1 ml of boiling 50 m*M* sodium ace-
uffer, pH 4.0, containing 2000 cpm of
yclic AMP to assess recovery (approx
). Cells were scraped into size AA
as homogenizers, homogenized, and
d at 100° for 10 min. The cooled ho-
nates were centrifuged at 3000 rpm for
in. Aliquots (50 μl) were then assayed
tly for cyclic AMP content by the bind-
rotein method of Gilman (19) as modi-
y Mashiter *et al.* (20). Cell pellets were
lved with 0.2 *N* NaOH and the protein
ntration was determined by the Lowry
in method (18). Data is expressed on
asis of total picomoles of cyclic AMP
late, but similar results are obtained on
milligram of protein basis.

ults. Six hours after addition of chol-
xin to CCL-6 cells, the cyclic AMP
nt dramatically increased by 85 ± 6
/plate (Table I, Expt. 1). Of the compounds tested, only 2′,5′-dideoxyadenosine and 2′-deoxyadenosine successfully suppressed cholera toxin-mediated increases in cyclic AMP content. 2′,5′-Dideoxyadenosine was the more potent inhibitor. As shown in Table I, Expt. 1, 2′,5′-dideoxyadenosine inhibited cholera toxin by nearly 70% while 2′-deoxyadenosine inhibited by only 25%. 2′,5′-Dideoxyadenosine was able to effectively inhibit cholera toxin from 5 to 60 min after addition to cholera toxin-treated cells; shorter and longer times were not examined.

The ability of 2′,5′-dideoxyadenosine to directly inhibit CCL-6 cell adenylate cyclase was examined next. At 500 μ*M*, the adenosine analogs tested caused a 95 to 20% inhibition of cholera toxin-stimulated adenylate cyclase activity (Table II). 2′,5′-Dideoxyadenosine was again the most potent inhibitor. Shown in Fig. 1 is the concentration-dependent inhibition of cholera toxin activation of adenylate cyclase by 2′,5′-dideoxyadenosine. Half-maximal inhibition was seen at 16 μ*M*. To determine the extent of 2′,5′-dideoxyadenosine inhibition of adenylate cyclase activity, the NaF-activated enzyme was also examined (Table

TABLE II. EFFECT OF VARIOUS ADENOSINE ANALOGS ON CHOLERA TOXIN-STIMULATED CCL-6 CELL ADENYLATE CYCLASE ACTIVITY.[a]

Additions	µM	Picomoles cyclic AMP formed/mg/10 min ± SEM
None		670 ± 8
2′,5′-Dideoxyadenosine	500	40 ± 10
Adenosine	500	354 ± 9
2′-Deoxyadenosine	500	180 ± 6
2-Chloroadenosine	500	550 ± 24

[a] Cells grown on 150-mm petri dishes were incubated with 0.7 µg/ml of cholera toxin. After 18 hr, membranes were prepared and exposed to the adenosine analogs during the 10-min assay of cyclase activity at 30°. The adenylate cyclase activity of cells not treated with cholera toxin was 18 ± 2 pmole cyclic AMP/mg/10 min. $N = 4$.

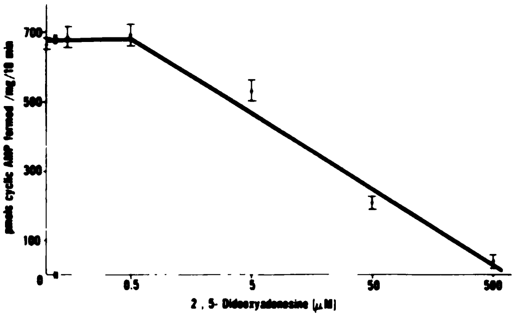

FIG. 1. Concentration-dependent inhibition of cholera toxin-stimulated CCL-6 cell adenylate cyclase by 2′,5′-dideoxyadenosine. Confluent cells were exposed to 0.7 µg/ml of cholera toxin at 37° for 18 hr. A membrane fraction was then prepared and cyclase activity determined in the presence of the indicated concentrations of 2′,5′-dideoxyadenosine $N = 4$.

III). As previously shown by Kantor *et al.* (21), NaF stimulated CCL-6 cell adenylate cyclase to a larger extent than cholera toxin. 2′,5′-Dideoxyadenosine inhibited both stimulated enzymes. There was greater inhibition of the cholera toxin than NaF-stimulated enzyme.

Discussion. This study demonstrates a potential usefulness of certain adenosine analogs in blunting the increases in intestinal cell cyclic AMP caused by cholera toxin. Of the adenosine analogs tested, 2′,5′-dideoxyadenosine was the most effective inhibitor. Similarly, Fain *et al.* (13) have shown this compound to be the most effective adenosine inhibitior of fat cell adenylate cyclase. Inhibition was not due to a nucleotide intermediate since 2′,5′ dideoxyadenosine is not suitable for such a conversion (22). Although CCL-6 cell adenylate cyclase activity has been shown to be elevated by 2 hr after addition of toxin (16), inhibition by 2′,5′-dideoxyadenosine in intact cells was observed at 6 hr after toxin addition, well after initial activation of adenylate cyclase occurs. Inhibition was apparent by 5 min after addition of inhibitor and could be sustained for at least 1 hr. This lowering in the cyclic AMP content of cholera toxin-treated cells could not be explained by excretion of cyclic AMP into the media since no change in the CCL-6 cell media cyclic AMP content was observed. Furthermore, in additional studies not shown, 2′,5′-dideoxyadenosine inhibition of cholera toxin was also observed in murine thymocytes where cells plus media were analyzed.

Inhibition of cholera toxin-mediated increases in cellular cyclic AMP content was reflected in a corresponding decrease in CCL-6 cell adenylate cyclase activity. The NaF-, as well as cholera toxin-, stimulated cyclase was inhibited. Differences in the mechanism of adenylate cyclase activation by NaF and cholera toxin have been reported (23–25) and are reflected in the insensitivity of the NaF-stimulated cyclase to 2′,5′-dideoxyadenosine inhibition compared to the toxin-stimulated enzyme. Various adenosine analogs have also been shown to inhibit adenylate cyclase in rat brain (12), liver (15), fat cells (13), and guinea pig lung (14). This supplementary evidence along with the high concentration of cyclic phosphodiesterase inhibitors pres-

TABLE III. EFFECT OF 2′,5′-DIDEOXYADENOSINE ON CHOLERA TOXIN- AND NaF-STIMULATED CCL-6 CELL ADENYLATE CYCLASE ACTIVITY.[a]

		Picomoles cyclic AMP formed/mg/10 min ± SEM	
Additions	µM	Cholera toxin	10 m*M* NaF
None		730 ± 50	1350 ± 94
2′,5′-Dideoxy-adenosine	500	83 ± 6	580 ± 55
	50	220 ± 12	870 ± 64

[a] Membranes were prepared from cells exposed to either 0.7 µg/ml of cholera toxin or diluent (saline) at 37° for 18 hr. 2′,5′-Dideoxyadenosine and NaF were present only during the 10-min cyclase assay at 30°. Adenylate cyclase activity of cells not treated with cholera toxin was 24 ± 2 pmole cyclic AMP/mg/10 min. $N = 4$.

ent during these experiments and the irreversibility of the cholera toxin binding suggest that the actions of 2′,5′-dideoxyadenosine are at least due in part to direct inhibition of adenylate cyclase and not activation of cyclic phosphodiesterase or dissociation of cholera toxin from its receptor. However, realization of the therapeutic usefulness of 2′,5′-dideoxyadenosine and related compounds awaits demonstration of the concomitant ability of these test agents to lower cholera toxin-mediated intestinal ileal cyclic AMP content and fluid accumulation.

Summary. The ability of various adenosine analogs to inhibit cholera toxin activation of the intestinal epithelial cell adenylate cyclase-cyclic AMP system was investigated. After incubation of cells with cholera toxin for 6 hr, large increases in cellular cyclic AMP content were observed. Addition of 2′,5′-dideoxyadenosine during the last 30 min of this 6-hr incubation resulted in 70% reduction in elevated cyclic AMP content. Other analogs were not effective inhibitors. 2′,5′-Dideoxyadenosine was also a potent inhibitor of cholera toxin-activated intestinal cell adenylate cyclase activity with half-maximal inhibition occuring at 16 μM. NaF-stimulated cyclase was less susceptible to inhibition. The data suggest that inhibition by 2′,5′-dideoxyadenosine is due at least in part to direct inhibition of the cholera toxin-activated intestinal adenylate cyclase activity.

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Serologic Response to BK Virus following Human Infection with SV40 (39343)

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Evidence was presented in a previous paper (1) suggesting that infection in man with BK virus, a recently discovered human papovavirus, could induce low levels of antibody to SV40. An opportunity to examine the converse question, as to whether infection in man with SV40 could induce antibody to BK virus, was made possible by the retrieval and testing of sera from a unique earlier study in volunteers infected with respiratory syncytial virus (RSV) which was later found to contain live SV40 (2).

Materials and methods. Paired sera were available from 34 of the original group of healthy adult males who had been given a nasal instillation of 10,000 $TCID_{50}$ of SV40, in addition to RSV with or without antiserum. In this earlier study, antibody to SV40 in pre- and postinfection sera, starting at a 1:5 dilution, had been determined in neutralization tests using 250 $TCID_{50}$ of SV40 in tubes of grivet kidney cells (2). Most of the postinfection sera had been obtained between 2 and 4 weeks after infection, but a few were collected as early as 10 days, or as late as 90 days, after infection. Sera were stored continuously at −20° since completion of this study in 1960. In the present study, aliquots of these sera were extracted with acetone to give an initial dilution of 1:20, and antibody to BK virus was determined in a hemagglutination-inhibition (HI) microtiter test, which is more sensitive than the neutralization test, and can detect antibody to BK virus in sera stored at −20° for at least 15 yr (1).

Results. As shown in Table I, 18 of the 34 subjects who had received SV40 became seropositive to SV40, with neutralizing antibody titers ranging from 1:5 to 1:80. None of these subjects showed any rise in HI antibody to BK virus, whether or not they had demonstrated pre-existing BK antibody. In one of them, who presumably had experienced a natural infection with BK virus shortly before being infected with SV40, the level of BK antibody fell from 1:2560 to 1:40 during a 3-month period following administration of SV40.

Discussion. A small but definite degree of antigenic sharing between SV40 and BK virus has been demonstrated in serological studies using hyperimmune animal sera. Employing the immune-electron microscopy test, a weak reaction has been observed between BK virus and SV40 antisera, and between SV40 and BK virus antisera (3, 4). A similarly weak reciprocal cross reaction between SV40 and BK virus has been shown to occur in fluorescent antibody tests (5). Antisera to BK virus also show low neutralizing activity against SV40 in plaque-reduction tests, and in tissue cul-

TABLE I. ANTIBODY RESPONSES TO SV40 AND BK VIRUS IN 18 HUMAN VOLUNTEERS INFECTED WITH SV40.

	BK virus HI antibody titer		SV40 Neutralizing antibody titer[a]	
Subject	Preinfection	Postinfection	Preinfection	Postinfection
No pre-existing BK antibody				
1	<20	<20	<5	5
2	<20	<20	<5	5
3	<20	<20	<5	5
4	<20	<20	<5	10
5	20	<20	<5	10
6	<20	<20	<5	20
7	<20	<20	<5	>20
Pre-existing BK antibody				
8	40	40	<5	5
9	40	40	<5	5
10	80	80	<5	5
11	40	40	<5	10
12	40	40	<5	10
13	80	40	<5	10
14	40	40	<5	>20
15	40	40	<5	>20
16	40	40	<5	>20
17	80	80	<5	80
18	2560	40	<5	80

[a] Data from original study (2).

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ture, BK virus induces T-antigen(s) immunologically similar to or identical with the SV40 T-antigen(s) (5). No cross reactions between the two viruses have been observed by HI (5), CF (3, 5), immunodiffusion (6), or immunoelectrophoresis (6).

These data raise the question of virus responsibility for the antibody patterns seen in man. Vaccine and monkey sources of SV40 infection are known to account for most of the SV40 antibody found in humans (7), and circumstantial evidence suggests that BK virus infection accounts for virtually all the rest (1). Evidence presented in the present study indicates that, in contrast, proven human infection with SV40, even in individuals with substantial homologous antibody responses, does not induce cross-reactive HI antibody to BK virus.

Summary. A small group of human volunteers given live SV40 virus, and who seroconverted to SV40, did not develop any heterologous antibody response to BK virus, even when BK antibody was demonstrated in preinfection sera. SV40 infection does not appear to contribute significantly to the patterns of BK antibody seen in human populations.

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Pleural Fluid Lysozyme in Human Disease[1] (39344)

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Despite the availability of knowledge concerning the pathophysiology and clinical usefulness of lysozyme (EC 3.2.1.17, mucopeptide *N*-acetylmuramylhydrolase, muramidase) of serum, urine, cerebrospinal fluid, middle ear fluid, amniotic fluid, genital secretions, breast milk, and synovial fluid in the diagnosis of malignant and infectious disorders (1-9), the content and source of lysozyme in pleural fluid remain poorly understood. In a preliminary study of a patient with recurrence of acute myelocyte leukemia involving pleura while bone marrow and blood were in remission, we found high lysozyme activity in the pleural fluid (10). This observation raised the possibility that measurement of the enzyme in pleural fluid might be useful in the diagnosis of pleural leukemia and other malignancies. Therefore, a prospective study was conducted to explore the potential diagnostic value of the content of pleural fluid lysozyme and to evaluate the source of the enzyme in the pleural fluid.

Materials and methods. Pleural fluid was obtained by thoracentesis from 59 patients admitted to University Hospitals. The pleural effusion was due to malignant neoplastic diseases in 37 patients and nonmalignant diseases in 22. The neoplastic group was composed of breast carcinoma in 7, lung carcinoma in 9, malignant lymphoma (including Hodgkin's disease and non-Hodgkin's lymphomas) in 9, acute myeloblastic leukemia in 2, undifferentiated carcinoma in 3, and leiomyosarcoma, carcinoid, mesothelioma, Waldenstrom's macroglobulinemia, malignant melanoma, chronic lymphocytic leukemia, and adenocarcinoma of the colon each in 1. The diagnosis of the malignancy was based on tissue biopsy in all patients. The non-neoplastic group included congestive heart failure in 10, liver cirrhosis in 3, pulmonary tuberculosis in 2, and miscellaneous conditions in 7. Paired serum samples were available from 50 of the 59 patients. These were obtained simultaneously or within 24 hr of the thoracentesis. The study was conducted prospectively. In addition to the routine diagnostic work-up, a protocol was designed to include the following determinations in each patient: (i) pleural fluid lysozyme, albumin, total protein, lactic dehydrogenase, granulocyte, total white blood cell, culture for bacteria and fungi, and cytology; and (ii) serum lysozyme, albumin, total protein, lactic dehydrogenase, white cell count, and differential. The differentiation of exudate and transudate was based on protein concentration and the ratio of pleural fluid and serum lactic dehydrogenase activity (11).

Lysozyme activity was measured by a modification (12) of a turbidometric method using egg white standard (13). The change in optical density at 20° of a cell-wall suspension of micrococcus lysodeikticus (Difco Laboratories, Detroit, Michigan) was measured at 540 nm on a recording spectrophotometer (Perkin-Elmer Coleman Instruments Division, Maywood, Illinois) at a chart speed of 20 mm/min. Initial optical density of the substrate was constituted to 0.91 ± 2 in phosphate buffer at pH 6.2. The assay is linear between 2.5 and 25 μg/ml. Samples with activity greater than 25 μg/ml were diluted. Normal serum lysozyme determined in our laboratory on 21 controls was 7.4 ± 0.43 μg/ml (mean ± standard error). Purified human lysozyme standards give values in our laboratory 0.30 times those obtained using egg white lysozyme. Protein, white blood count, lactic dehydrogenase, creatinine, and cytology were performed by the University Hospitals Laboratory Service using standard techniques.

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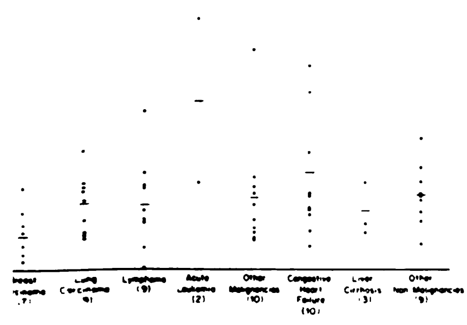
sults. The distribution of pleural fluid zyme activity in 59 patients with malig- ies and other medical diseases is shown ig. 1. The mean pleural fluid lysozyme ity was not significantly different ng various disorders studied. There was ignificant difference between the malig- effusions (12.17 ± SEM 1.50) and nalignant effusions (12.95 ± 1.82). wise there was no difference between sudates (11.86 ± 1.39) and exudates 23 ± 2.22).

ie correlation between the pleural fluid serum lysozyme concentrations in d samples from 50 patients was high a correlation coefficient (*r*) of 0.79 2). In contrast, the correlation be- n pleural fluid lysozyme levels and ral fluid granulocyte concentrations lable on 35 patients) was low with *r* = . Similar relationships were noted when omparisons were made only for those nts whose pleural effusion was due to gnant disease. There was a high correla- between pleural fluid and serum lyso- concentrations (*r* = 0.80) and low lation between pleural fluid enzyme granulocyte concentrations (*r* = 0.35). periments on four pleural fluid samples rmed by mixing fluid (20-40%) with a yme standard revealed no evidence of a ally occurring inhibitor in the pleural .



1. Pleural fluid lysozyme in malignant and lignant disorders. Parentheses enclose the num- patients in each group.

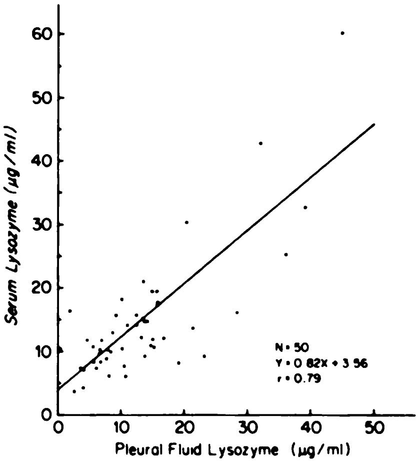


FIG. 2. Lysozyme activity in paired serum and pleural fluid samples.

cussion. The present study shows that leural fluid lysozyme activity correlated with simultaneous serum activity. Fur- iore, the activity of the enzyme had correlation with disease states, pleural fluid granulocyte counts, and total pleural fluid white blood cell counts. The relationship between pleural fluid and blood lysozymes appears similar for both the transudates and exudates. The indirect evidence from this study suggests that pleural fluid lysozyme may be derived primarily from the blood rather than the product of inflammatory or neoplastic cells in the pleural fluid. This may be explained on the basis of the low molecular weight of lysozyme (14,000-15,000) and its readiness to diffuse into the pleural space from the circulation. Previous studies have demonstrated a lack of correlation of tear (14) and synovial fluid (9) with plasma lysozyme. This may be the result of active transport, production, or release of the enzyme by lacrymal gland or inflamed synovium. There was no evidence of a similar mechanism involved in the determination of pleural fluid lysozyme activity.

Summary. A prospective study was conducted to define the content, significance, and source of lysozyme present in the pleural fluid in human diseases. The pleural fluid lysozyme activity is similar in various malignant and nonmalignant transudates and exudates, and is of limited diagnostic value. The pleural fluid activity correlated well with that of paired serum samples but it had poor correlation with the disease state, the pleural fluid granulocyte counts, and total white blood cell counts. The data suggest that the pleural fluid lysozyme may be derived primarily from the blood and that it

is not the product of inflammatory or neoplastic cells in the pleural fluid itself.

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tin Associated with Membranes of Monoamine Storage Organelles (39345)

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omyosinlike proteins occur in various
s other than muscle, e.g., in adrenal
lla (1, 2), neuronal elements (3), and
platelets (4, 5). It has been suggested
n brain synaptosomes stenin (myosin-
and neurin (actinlike) are localized in
resynaptic vesicles and the synaptic
ranes respectively (3, 6), but this sub-
ar distribution remains to be demon-
d.

ently, specific antiactin autoantibod-
AA) binding to frozen sections of
th and striated muscle as well as to
ets have been described in the serum
ients with chronic aggressive hepatitis
The use of such antibodies and the
nstration of filaments 40–80 Å in di-
r by electron microscopy has been
in demonstrating the presence of ac-
several nonmuscular cells (8–10).

vas therefore of interest to investigate
eans of immunofluoresence using
serum whether actin could be local-
n isolated amine storage organelles of
al medulla and blood platelets.

thods. Homogenates of bovine adrenal
lla and rabbit blood platelets were sub-
d to ultracentrifugation in a sucrose
on (11) and an Urografin gradient,
ctively, as previously described (12,
The majority of the organelles storing
olamines (adrenal medulla) or 5-hy-
tryptamine (5HT) (platelets) sedi-
d as a fine layer at the bottom of the
(fraction 10). The supernatants were
d into nine equal portions, each of
was diluted 1:1 with 0.3 *M* sucrose,
ltracentrifuged for 30 min at 180,000
rder to obtain the particulate matter
Furthermore, portions of the bottom
(fraction 10) were lysed by twofold
ng and thawing in distilled water. The
ranes of the lysed organelles were
sedimented by ultracentrifugation (15). Intact amine storage organelles and their membranes as well as the particulate matter of the other fractions were submitted to electron microscopy and processed for the immunofluorescence assay. Another portion of the chromaffin granules was lysed in hypotonic KCl (0.015 *M*) followed by dialysis against 4 liters of 0.15 *M* KCl (16). The membranes were sedimented by ultracentrifugation (180,000 *g* for 30 min) and also examined for immunofluorescence.

For electron microscopy, the material was fixed in an ice-cold 3% solution of glutaraldehyde in cacodylate buffer, pH 7.4, postfixed in 2% solution of osmium tetroxide in the same buffer at 4°, dehydrated in graded concentrations of alcohol, and embedded in Epon 812. Ultrathin sections stained with uranyl acetate and lead citrate were examined with a Philips 300 electron microscope.

The immunofluorescence reaction was performed on 4-μm sections obtained by cutting the frozen material on a cryostat. After treatment for 30 min with AAA serum, followed by washing in phosphate-buffered saline (PBS), the sections were stained for 30 min with a fluorescein-conjugated IgG fraction of goat antiserum to human IgG (Miles Seravac, Lausanne, Switzerland, Code 64.170). The intensity of fluoresence of the sections (rewashed with PBS and mounted in 90% glycerol in PBS) was compared with that of control sections treated with normal human serum. The AAA-containing sera were obtained from two patients with chronic aggressive hepatitis (7); their titers (judged by immunofluorescent staining of rat intestinal smooth muscle) amounted to 1/1280 and 1/640, respectively. Tests for antimitochondrial or anti-DNA antibodies as well as for antinuclear factor were negative (for details, see 7). Photographs were taken on Zeiss 50/65 barrier filter using Anscochrome color slide

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film 500 daylight (Gaf Co., New York, N. Y.).

Results and discussion. In agreement with previous results, the electron micrographs of the bottom layers of the centrifugation tubes (as well as of the pellets from fractions 7, 8, 9 of the adrenal medullary homogenate) showed a population of virtually pure storage organelles with electron dense, osmiophilic cores due to the presence of high concentrations of catecholamines (Fig. 1c) and 5HT (14). After lysis of the organelles, vesicular membranes without or with little osmiophilic contents were seen (15). The particulate matter of the other fractions was very poor in amine storage organelles, but contained other subcellular elements, e.g., in adrenal medulla microsomes (fractions 1-2, Fig. 1a) and mitochondria (fraction 4, Fig. 1b) and in platelet α-granules (fractions 7, 8).

Treatment of sections of intact adrenal chromaffin storage vesicles (fraction 10) with AAA serum followed by staining with the fluorescein conjugated anti-human IgG fraction gave rise to an intense fluorescence throughout the preparation with a few bright spots probably due to irregular distribution of the organelles (Fig. 2b). A similar fluorescence pattern was seen with the vesicular membranes obtained by freezing and thawing the isolated storage organelles in

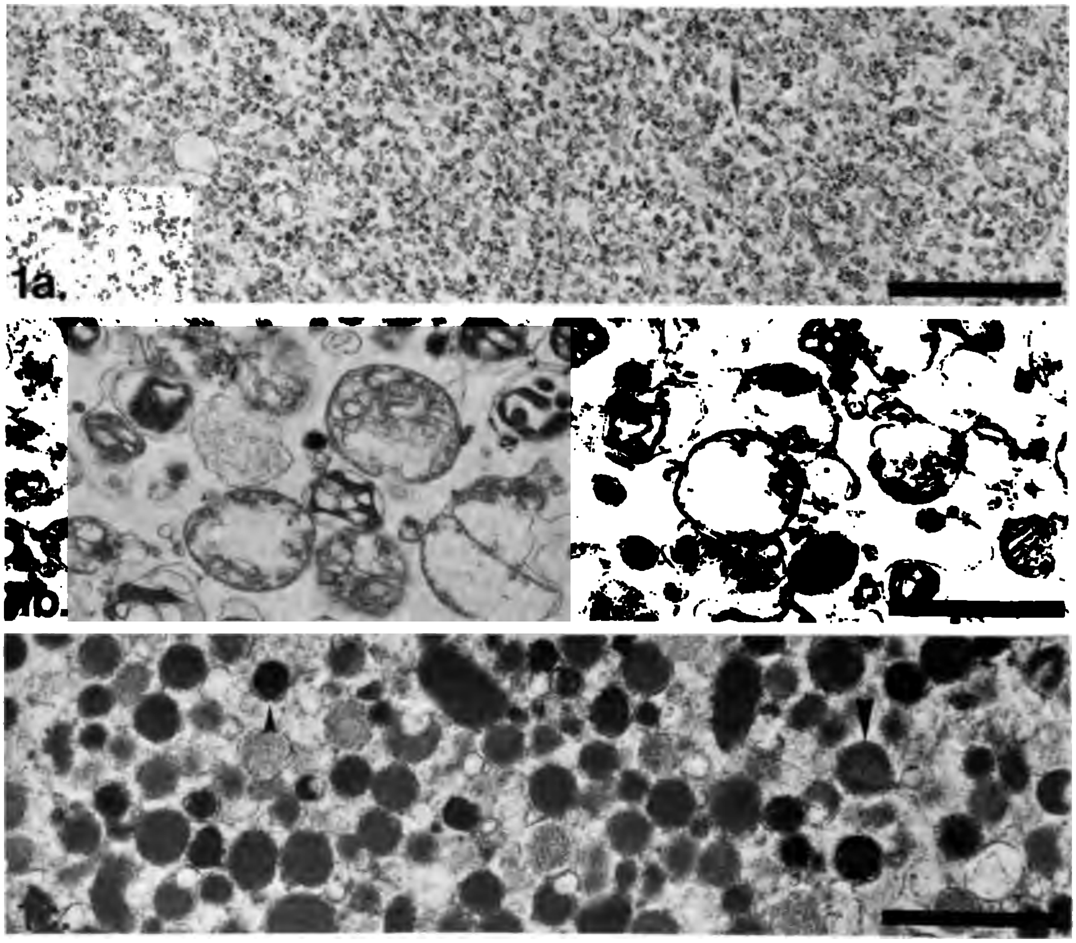

FIG. 1. Ultrastructural aspects of subcellular fractions of bovine adrenal medulla. (a) A microsomal pellet (fraction 2) is virtually pure in microvesicles and contains no chromaffin granules. (b) A crude mitochondrial pellet (fraction 4) is a mixed population of organelles containing very few chromaffin granules. (c) A chromaffin granule pellet (fraction 10) contains a high density of amine storage organelles. ▶, Noradrenaline storage vesicle; ▶, adrenaline storage vasicle. Bar 1 μm. Magnification: × 22,000.

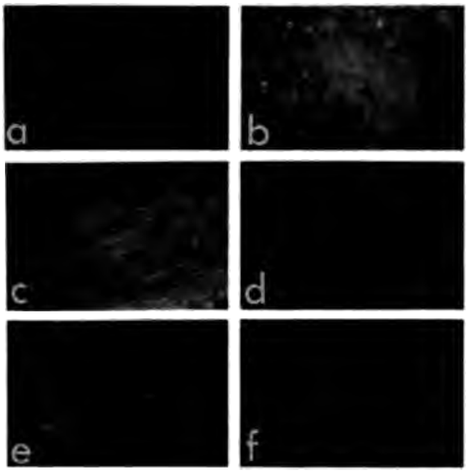

FIG. 2. Immunofluorescent staining of cryostat sections of different cellular fractions from adrenal medullary cells isolated by ultracentrifugation. (a) Intact storage organelles incubated first with normal human serum followed by goat anti-human IgG: no staining (× 400). (b) An adjacent section incubated first with AAA followed by fluorescent goat anti-human IgG: bright fluorescence is seen throughout the section with few brighter spots randomly distributed (× 400). (c) Storage organelles lysed after twofold freezing and thawing in distilled water stained as in (b). The intensity and the distribution of fluorescence are similar to that seen with intact granules (× 400). (d) Storage organelles lysed in 0.015 *M* KCl, treated as in (b): no staining (× 400). (e) Mitochondrial and microsomal fraction isolated from the adrenal medulla and treated as in (b). A very faint stain is present, much less intense than that seen in fractions of intact or frozen and thawed granules (× 400). (f) Intact storage granules incubated first with AAA absorbed with thrombostenin A, followed by goat anti-human IgG: no staining (× 400).

distilled water (Fig. 2c). Frozen sections of particulate matter from fractions containing microsomes and mitochondria (Fig. 1a, b) did not show relevant fluorescence after treatment with AAA (Fig. 2d).

The specificity of the immunofluorescence reaction is indicated by the following findings: (a) When sections of storage organelles were treated with AAA serum which had been preincubated with highly purified thrombostenin (17) (kindly supplied by Prof. E. F. Lüscher, Theodor-Kocher Institute, Berne, Switzerland) or rabbit skeletal muscle actin (18), no immunofluorescence was seen on exposure to the fluorescein conjugated IgG fractions (Fig. 2f). In contrast, sections treated with AAA serum preincubated in the buffers alone developed marked fluorescence. (b) Dialysis of the isolated storage organelles in 0.015 *M* KCl markedly decreased the immunofluorescence reaction of sections of the vesicular membranes (Fig. 2e). This is probably related to the previously demonstrated solubility of actin in KCl (19) leading to a washout of this protein from the membrane.

It has to be considered that during the homogenization and fractionation procedures actomyosin might have precipitated and cosedimented with the chromaffin granules at the bottom of the tube. This possibility was examined by putting an aqueous suspension of platelet actomyosin (isolated according to Probst *et al.* (17)) on a 1.6 *M* sucrose solution identical to that used for the isolation of the amine storage organelles (11). After centrifugation (45 min at 142,800 *g*), the actomyosin determined colorimetrically (20) equilibrated close to the top of the sucrose solution, and no sediment was found at the bottom of the tube.

Isolated 5HT organelles and their membranes from rabbit blood platelets also exhibited intense fluorescence on exposure to AAA. The immunofluorescence reaction showed the same specificity for actin as that observed with chromaffin granules. However, a marked fluorescence was also present in the fractions containing α-granules and mitochondria and, as observed earlier, in frozen sections of whole platelets throughout the cytoplasm (7). This indicates that in platelets actin is not only connected with membranes of 5HT organelles, but also with other subcellular structures.

It is noteworthy that, contrary to platelets (7), frozen sections of bovine adrenal medulla did not show significant immunofluorescent staining after treatment with AAA. This indicates that the overall amount of actin present in the cytoplasm of medullary cells is probably not sufficient in order to be visualized with the immunofluorescence technique. Isolation of the storage organelles by ultracentrifugation appeared to result in a concentration of actin, as shown by immunofluorescence. On the other hand, isolation of mitochondria or microsomes was not accompanied by concentration of the protein.

Recently, it has been reported that

chicken actin and myosin mixed with bovine adrenal chromaffin granules adhere to their membrane, having two independent attachment points (21). The present results indicate that, during the isolation procedure, endogenous actin remains firmly associated with the membranes of amine storage organelles. This association appears rather specific in the adrenal medulla, but not in the platelets, and may indicate a direct or indirect function of contractile proteins in catecholamine release and possibly in the release reaction of platelets.

The nature of the events leading to monoamine liberation, however, is not yet known. Actin, in relation with vesicular membranes, may interact with myosinlike proteins and, as suggested before (6, 22, 23), may lead to the fusion of vesicular and plasma membrane resulting in exocytosis. In addition, it has to be considered that the actomyosin system has a function in actively expelling the amines from the storage vesicles during the process of release (24).

Summary. A histo-immunofluorescence technique using anti-actin antibodies has been applied to various subcellular fractions of bovine adrenal medulla and rabbit blood platelets. In the adrenal medulla only the membranes of the chromaffin granules, but not the fractions containing other subcellular particles (microsomes, mitochondria) showed marked immunofluorescence. In platelets, considerable fluorescence was present in the membranes of the 5-hydroxytryptamine organelles as well as in other subcellular particles. It is concluded that in the adrenal medulla, actin is specifically associated with the membranes of the amine storage organelles, whereas in platelets the protein shows a rather general subcellular distribution.

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Curie	Ci	molar (concentration)	*M*
degree Celsius (Centigrade)	-°	mole	spell out
degree Fahrenheit	°F	molecular weight	mol wt
diameter	dia	nanogram	ng
gram	g	nanometer	nm
hour	hr	normal (concentration)	*N*
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inside diameter	i.d.	ounce	oz
intramuscular	im	outside diameter	o.d.
intraperitoneal	ip	parts per million	ppm
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liter	spell out	second	sec
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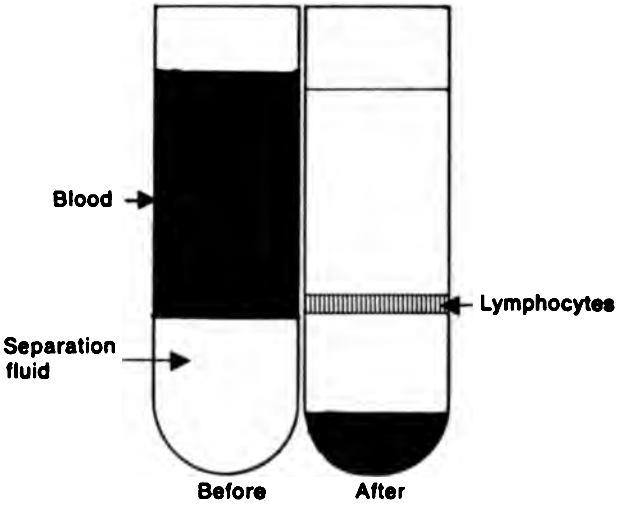

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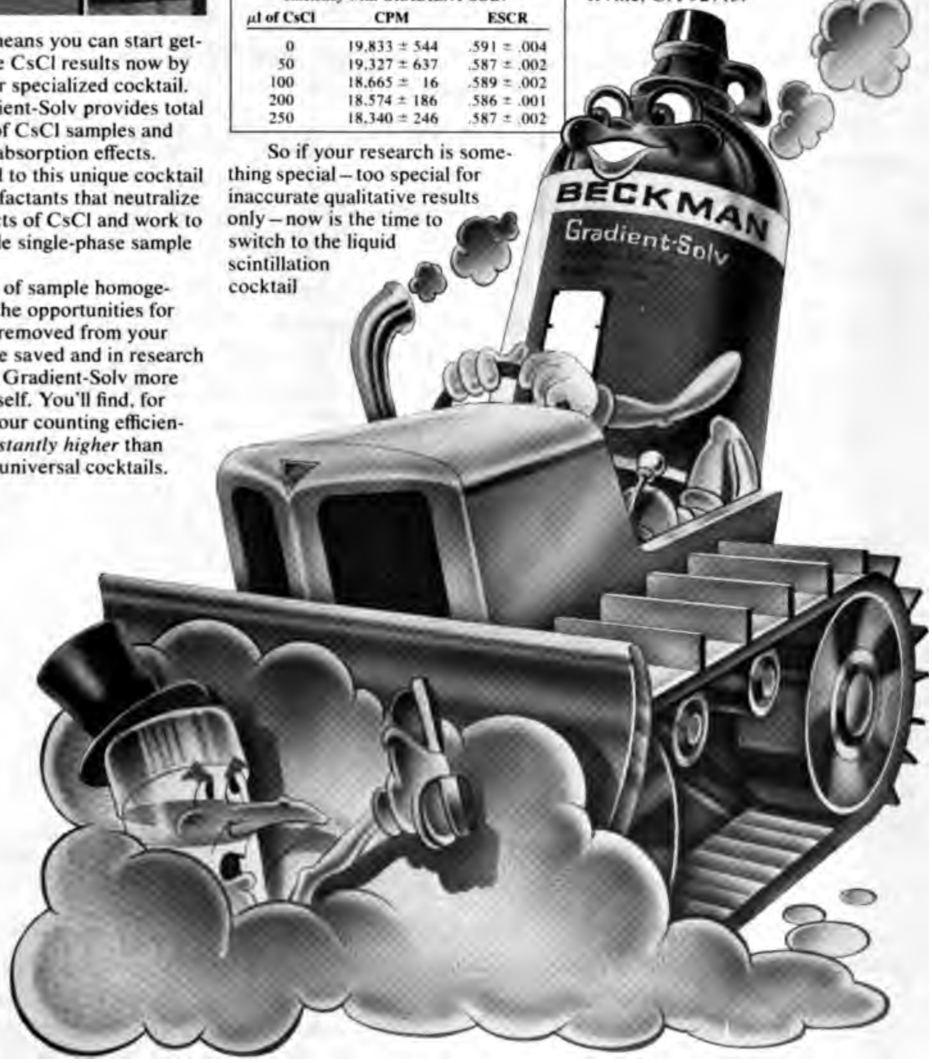

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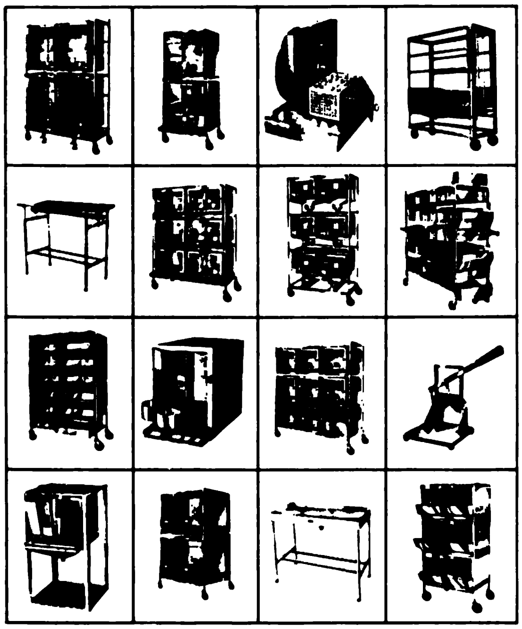

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OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 139-142 (1976)

assium Cyanate, An *in Vitro* Inhibitor of Lymphocyte Blast Transformation without *in Vivo* Activity[1] (39346)

HUR I. GRAYZEL, EUGENE F. ROTH, JR., AND CAROLYN L. BECK

ent of Medicine, Montefiore Hospital and Medical Center, and the Albert Einstein College of Medicine, Bronx, New York 10467

ability of cyanate to react with pro-
as been extensively studied in recent
ollowing reports of its capacity to
erythrocyte sickling *in vitro* (1).
of the cyanate reaction with hemo-
have shown that the N-terminal
groups of the B-chain are carbamy-
irst, but at higher concentrations of
many other amino groups react as
). Since protein carbamylation is a
and nonspecific reaction, cyanate
n studied for its effects on other cell
ents, as well as for its toxicity to
organ systems *in vivo*. Alter *et al.*
nd definite evidence of depressed he-
in synthesis in red cell precursors
with cyanate, and long term studies
emonstrated severe toxicity in both
and animals treated chronically
anate doses over 35 mg/kg/day. In
, peripheral neuropathy and cataract
on (4) have been the most promi-
mptoms of toxicity.

e findings have led some workers to
cyanate for the extracorporeal
nt of blood to avoid exposing other
rgans. In one ongoing study Har-
nd Diederich (5) exposed blood for 4
7° to 50 m*M* cyanate in a dialysis
e which resulted in the carbamyla-
hemoglobin to the extent of 2.3 mole
ate per mole of hemoglobin tetra-
hese methods expose all blood con-
s to cyanate and carbamylate many
f proteins in cells and in the plasma.
lating agents, of which cyanate is
e well known for selective toxicity
lymphocytes while the prototype of
gents, nitrogen mustard, has been
to inhibit the sickling of red blood
vitro (6). Protein synthesis is an early, essential component of the lymphocyte response to mitogens. It was of interest, therefore, to examine the effect of exposure to cyanate on the cellular arm of the immune response.

Methods and materials. Isolation of lymphocytes and PHA stimulation. Blood was obtained from laboratory personnel or from apparently healthy blood bank donors. In a typical experiment 40 ml of blood was collected in sterile plastic centrifuge tubes containing 10,000 USP units of heparin in isotonic saline (Panheprin, Abbot Laboratories, North Chicago, Ill.). Lymphocyte-enriched plasma was obtained by adding 5 ml of 5% dextran in saline with an average molecular weight of 200,000 (Poviet Prodeuctin, Amsterdam, Holland) and allowing the erythrocytes to sediment for 30 min at 37°. Nylon fiber columns were made by filling a 30-ml glass syringe with nylon wool (Scrub nylon fiber ST-242, Fenwall Laboratories, Morton Grove, Ill.) washed with 1 liter of distilled water and sterilized. The columns were then washed with a small amount of tissue culture medium and the leukocyte-enriched plasma mixed with an equal volume of prewarmed medium, was passed over the column at a flow rate of 1 ml per min or less at 37°. The column was washed with additional tissue culture medium and the combined eluate containing lymphocytes and a variable but small number of erythrocytes and platelets was centrifuged at 400 rpm for 10 min. The loosely packed cells were diluted in tissue culture medium and counted in a hemocytometer.

The medium used in all experiments was Dulbecco-Vogt modified Eagle's medium containing 10% calf serum, and all incubations were carried out in a total volume of 1 ml containing 10^6 lymphocytes. Phytohemagglutinin P (Difco Laboratory, Detroit, Mich., lot number 575119) was put into

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solution prior to each experiment and used in a final concentration of 250 μg/ml. Lymphocytes were pretreated with potassium cyanate (Eastmen Kodak, Rochester, N.Y., and recrystallization from ethanol) at a concentration of 400 μg/ml, unless otherwise specified, and incubated for 30 min at 37°. They were then washed twice with tissue culture medium and resuspended to a final concentration of 10^6 per ml. Control cells were initially treated with an equivalent amount of potassium chloride. Phytohemagglutinin was then added and the culture tubes were loosely capped and incubated under an atmosphere of 5% CO_2 in room air for 3 or 7 days. At the end of the incubation period, lymphocyte viability and blast transformation were assayed by mixing a drop from each culture tube with a drop of freshly dissolved acridine orange 2.5 μg/ml in isotonic saline and by examining the cells under ultraviolet illumination (7). The nuclei of viable cells appeared bright green and blasts were identified by their size and orange cytoplasm. On Day 3, 0.125 μCi of [^{3}H]thymidine, 16 Ci/mmol (Schwarz Bioresearch, Orangeburg, N.Y.) was added for a period of 18 hr. The following day, the contents of each tube were collected on Whatman GF-C glass fiber filters (8). The tubes were rinsed several times with saline which was added to the filter. The filters were then washed with 50 ml of cold isotonic saline followed by 25 ml of cold 5% trichlorocetic acid (TCA) and 25 ml of methyl alcohol. The filters were placed in glass liquid scintillation vials and 0.5 ml of hyamine was added. The vials were stoppered and incubated at 37° overnight. The following morning 10 ml of toluene scintillator was added and the vials counted.

Mixed lymphocyte culture reactions were performed as one-way reactions. The stimulating cells were treated with 50 μg of mitomycin C (Schwarz Mann, Orangeburg, New York) for 30 min. The cells were then washed twice with tissue culture medium and resuspended. Both stimulating and responder cells were treated with mitomycin to determine base incorporation values. Responder cells were pretreated with potassium cyanate or potassium chloride as described above. The cultures were allowed to incubate for 5 days. At the end of this time, the percentage of blasts was counted and viability assessed by means of acridine orange staining, and the incorporation of tritiated thymidine was measured as described above.

Results. Cyanate was noted to inhibit blast transformation in response to PHA as examined by morphologic change and the incorporation of [^{3}H]thymidine. More importantly 400 μg/ml of cyanate virtually abolished the response of lymphocytes to allogeneic lymphocytes (Table I). The values for each experiment represent the mean of triplicate culture tubes.

The dose of cyanate, 400 μg/ml, was chosen because it is equivalent to the 5 m*M* (405 μg/ml) dose demonstrated to inhibit erythrocyte sickling *in vitro*. Cyanate alone did not reduce thymidine incorporation in nonstimulated lymphocytes by more than 5%. The addition of 400 μg/ml of KCl to stimulated or unstimulated lymphocytes had no effect on [^{3}H]thymidine incorporation. Sodium cyanate was equally effective on a weight basis in inhibiting the PHA response. In MLC experiments the reverse experiment, namely the exposure of the stimulating or mitomycin-treated cells to KCNO for 30 min, had no effect on the response of untreated lymphocytes.

In view of the effect of cyanate on the *in vitro* response of lymphocytes in mixed lymphocyte culture, we decided to see whether cyanate was immunosuppressive *in vivo*. Some data were available on the pharmacology of cyanate in mice. Using a treatment schedule shown to produce significant carbamylation of hemoglobin in mice (9) we studied the ability of cyanate-treated animals to reject a first set skin allograft.

Brown CBA/J female mice obtained from Jackson Laboratories, Bar Harbor, Maine, were grafted with the skin obtained from white A/J mice. Twenty recipient mice with a mean weight of 17.52 ± 1.74 g were each given 525 μg of sodium cyanate (Pfaltz & Bauer, Flushing, N.Y.) by intraperitoneal injection with an average dose of 29.96 μg per gram body weight. Injections were given every 5 out of 7 days for a total of 16 injections as described by Cerami (9). The control CBA/J mice were given normal sa-

LE I. Inhibition of PHA-Induced Blastogenesis and the MLC Reaction by Cyanate.[a]

Percentage blasts		Counts per minute[b]					
PHA	PHA-KCNO	PHA	PHA-KCNO	Control[c]	MLC Ax Bm	MLC-KCNO A_{KCNO} × Bm	Control Am × Bm
78	4	30,867	9,332	247	15,560	998	396
70	2	5,585	567	182	2,136	187	110
74	3	60,196	1,710	510	7,769	173	265
78	13	101,190	30,383	9,481	9,726	506	265
70	6	40,810	3,630	1,380	4,705	325	182
68	5	19,690	3,740	367	9,269	166	138
73	5.5	41,389	8,227	2,027	9,246	392	226
±4.3	±4.9	±14,823	±4,694	±1,532	±2,106	±135	±44

'esponder cells (A) versus mitomycin-treated stimulator cells (Bm).

ılue represents the mean of triplicate incubation tubes.

:ed cells; cells treated with KCNO alone were included in each experiment but the values were not different from untreated cells.

ıe end of this time, skin grafts were ḋ and injections of cyanate or sa- given an additional seven times. treated and nine untreated mice and all mice, in both groups, re- :ir grafts on Days 10 or 11.

ion. The exact means by which ıhibits lymphocyte activation *in vi-* :ure, but one can speculate that it y carbamylating a protein involved ›cess that results in blast transfor- 'he failure to demonstrate an effect ay be dose related since 7.5% of a ^{14}C-labeled cyanate given to a as bound to the amino terminal hemoglobin, and only 0.01% was :he spleen, while lymph nodes and ɔd cells were not specifically exam-). Furthermore, Nehlsen and Lale- ıave demonstrated prolongation of ;rafts in mice following the daily of sodium cyanate at approxi- ı μg/g of body weight, a dose they to cause moderate toxicity. It is ble that a less stringent test of *in* ɹnity than allograft rejection might I by cyanate administration.

nces between the *in vitro* and *in* ity of cyanate have been observed ty of biological processes. For ex- m*M* cyanate inhibits hemoglobin n isolated human reticulocytes but y does not inhibit erythroid cell n nor produce a lack of hemoglo- › (11). In addition to these discor- ts with cyanate apparent contradictions between *in vitro* and *in vivo* effects have been noted in studies on lymphocytes involving other agents. Aspirin, for example, has been reported to inhibit lymphocyte transformation *in vitro* but contradictory results have been obtained following its administration *in vivo* (12–16). A drug may be more toxic *in vitro* due to the absence of detoxifying or excretory mechanisms offered by the liver and kidney *in vivo*.

At the present time an extracorporeal method of cyanate treatment for sickle cell anemia is being considered in which the *in vitro* model of drug usage is more relevant. Blood will be exposed to high concentrations of cyanate for a short time, then cyanate will be removed by cell washing or dialysis and the blood reinfused. Our data suggest that lymphocytes exposed to this amount of cyanate may show a diminished response to both specific and nonspecific antigens. A large pool of lymphocytes residing outside the vascular compartment, in the lymph nodes, spleen and thymus, and bone marrow, will not be affected initially. With chronic, intermittent treatment, however, very many more lymphocytes will be exposed to cyanate. Our data do not bear directly on the immunologic status of such an individual, but they do suggest caution in the treatment of whole blood *in vitro* with cyanate until appropriate studies are done.

Summary. Potassium cyanate (400 μg/ml) inhibited the blastogenesis of lymphocytes in response to PHA and the response of lymphocytes in mixed lymphocyte cul-

tures. Cyanate administered to mice at a dose of 30 mg per kg per day did not delay allograft rejection.

The authors wish to thank Dr. Sandra Nehlsen for help with the skin grafting.

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OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **152**, 143-146 (1976)

kade of the Postorchidectomy Increase in Gonadotropins by Implants of Atropine into the Hypothalamus (39347)

C. LIBERTUN[1] AND S. M. MCCANN[2]

nt of Physiology, The University of Texas Health Science Center at Dallas, Southwestern Medical School,
Dallas, Texas 75235

ine sulphate can suppress gonado-
elease in rats of both sexes following
utaneous (1-3) or intraventricular
n (2, 3). The aim of the present
as to localize the regions involved in
king action of atropine by implant-
nall amount of the alkaloid in dis-
ain regions.

ial and methods. Adult male rats
20 g body wt) obtained from Simon-
oratories (Gilroy, Calif.) were main-
n an air-conditioned room with con-
lighting (on 5 AM, off 7 PM). Purina
w and water were provided *ad libi-*

ether anesthesia, stainless steel
e (23-gauge, 20 mm in length) were
ed bilaterally in various hypotha-
ci or into the anterior pituitary using
ig Johnson stereotaxic instrument.
ae had been previously filled with
ture to be implanted by tamping the
the mixture. This mixture consisted
al amounts of atropine sulphate
mixed with cholesterol (w:w) (50%
), 1 part of atropine to 19 parts of
rol (5% atropine) or pure choles-
a control. Cannulae were implanted
e of the following loci: suprachias-
nterior hypothalamic region; the
hypothalamus, which was in the vi-
f the arcuate median eminence re-
he posterior hypothalamus, in the
mmillary and mammillary regions;
tal cortex at a depth of 2 mm from
a; or into each lobe of the anterior
y. Cannulae were secured to the
rface with dental cement.

Then, a 1-ml blood sample was withdrawn from the external jugular vein and this was followed by bilateral orchidectomy via the scrotal route at 4:30–6:30 PM. Sixteen hours later (8:30–10:30 AM on the following day) a second blood sample was withdrawn as before while the animals were etherized.

Verification of the location of the tips of the cannulae was accomplished readily by examining the base of the brain at autopsy and in some instances these localizations were confirmed after fixing the brain in 10% formalin and cutting serial frontal sections through the hypothalamic region at 20 μm which were then stained with cresyl violet. To measure the amount of drug delivered from the cannulae, they were filled in the routine manner and then using a mandril, the drugs were pushed out of the cannula and weighed. At sacrifice, the amount of drug remaining in the cannulae was similarly weighed so that the quantity delivered while the cannula was implanted could be estimated.

LH in plasma was measured by the method of Niswender *et al.* (4) and FSH using the kits provided by NIAMDD.[3] Results were expressed in terms of NIH-LH-S1 in the case of LH and the RP1 reference preparation in the case of FSH. The paired Student's *t* test was used to calculate the significance of changes in hormone titers produced by a treatment. When the means of two groups were compared, analysis was by Student's *t* test.

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gentina.

orted by USPHS grants (AM 10073 and HD
nd grants from the Ford Foundation, and the
oulation Research Institute.

[3] Antiovine LH serum was provided through the courtesy of Dr. Gordon Niswender (Colorado State University). Dr. Leo Reichert (Emory University) generously provided the purified ovine LH for radioiodination. Kit for the determination of prolactin was provided through the NIAMDD NIH Pituitary Hormone Program.

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erved.

Results. Cerebral cortex. Implants containing 50% atropine sulphate in the frontal cortex failed to block the increase in plasma LH and FSH titers which follows castration in the male rat (Figs. 1 and 2).

Anterior hypothalamic implants. Implants of cholesterol in the suprachiasmatic-anterior hypothalamic region were associated with elevations in both plasma LH and FSH following castration which were similar to those observed in animals with atropine implants in the cerebral cortex. In contrast, implants of 50% atropine sulphate completely abolished the postcastration rise of both LH (Fig. 1) and FSH (Fig. 2). The increase in both gonadotropins in this group was significantly less than the increase in the animals bearing cholesterol implants ($p < 0.05$). The implants of 5% atropine sulphate had only a slight and nonsignificant suppressive effect on the postcastration rise in both gonadotropins.

Mid-Hypothalamic implants. Results in this group of animals were similar to those in animals bearing anterior hypothalamic implants in that a postcastration rise in both gonadotropins occurred in cholesterol-implanted controls and no significant rise occurred in animals bearing implants of the higher but not the lower dose of atropine sulphate.

Posterior hypothalamic implants. Results in this group were also similar to those in the other hypothalamic groups except that there were no significant rises in plasma FSH in any of the implanted groups in this series. In the case of LH a postcastration rise occurred in animals implanted with cholesterol or the lower dose of atropine but no significant increase occurred in animals implanted with 50% atropine sulphate.

Intrapituitary implants. A significant rise in LH occurred in the castrates with implants of either cholesterol or the high dose of atropine in the anterior pituitary, but FSH failed to rise significantly following implantation of either cholesterol or atropine into the pituitary.

Quantity of atropine delivered by the implants. Weighing the material within the cannula before and after implantation showed that approximately 50 μg disappeared from each cannula or a total of 100 μg from both cannulae. In the case of 5% atropine implants this would mean that 5 μg of atropine sulphate was delivered to the brain. Since less than one-half of the weight of atropine sulphate is atropine, only about 2.2 μg of atropine would be released from the 5% atropine sulphate cannulae during the 16-hr period, a dose of 130 ng/hr. The quantity of atropine released in the case of

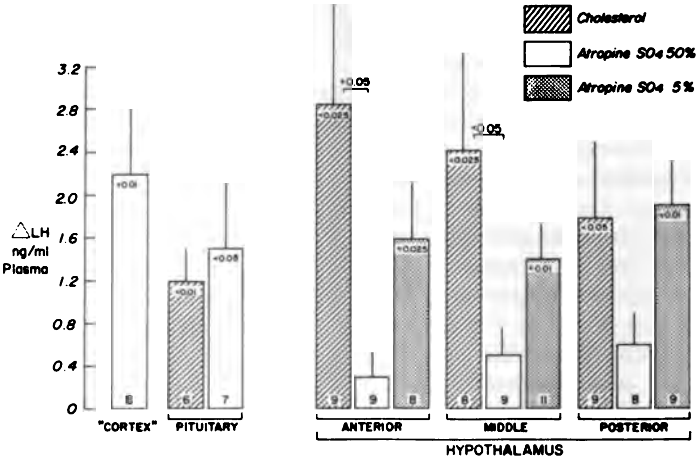

FIG. 1. The change in plasma LH 16 hr after castration in rats bearing implants of cholesterol or atropine sulphate in various hypothalamic loci, the cerebral cortex, or the anterior pituitary. In this and the subsequent figure, the height of the bar indicates the mean response and the vertical line 1 standard error of the mean. Significance of the change from the precastration plasma titer is indicated within each bar, and the number of animals is indicated at the bottom of each column.

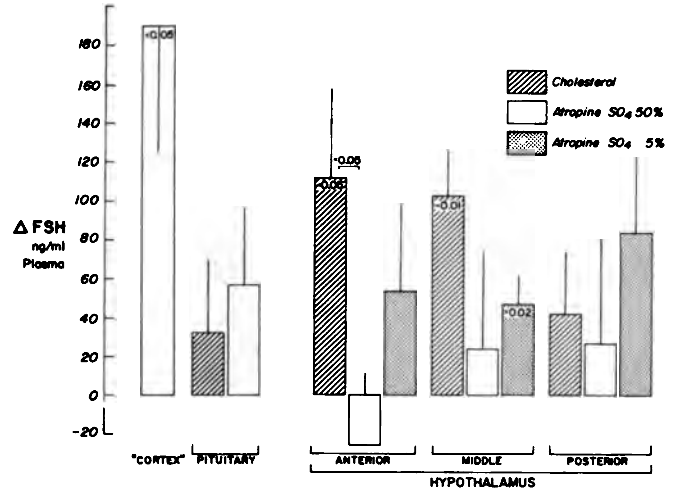

FIG. 2. The increase in FSH titers in plasma following castration in rats bearing various implants within the brain or pituitary.

the 50% atropine sulphate implants would be 1.3 μg/hr.

Discussion. The present results indicate that a small amount of atropine sulphate delivered to the basal hypothalamus can abolish the postcastration rise in both LH and FSH. On the other hand, implants within the cerebral cortex or pituitary failed to block the increase in plasma LH. It was not possible with this technique to localize definitively the most sensitive area within the hypothalamus since implants were effective whether implanted in the anterior, middle, or posterior hypothalamus; however, the most dramatic inhibition was found with implants in the anterior and middle hypothalamus which is the region in which changes in acetylcholine metabolism can be related to altered gonadotropin release (5). The ability of implants in such a wide area of the hypothalamus to inhibit the postcastration rise in LH may be related to diffusion of the atropine to a considerable distance from the cannula tip. Our results are in agreement with earlier work in which atropine implants into the anterior and middle hypothalamus were found capable of blocking ovulation in female rats (6), and it is noteworthy that similar intrahypothalamic implants of atropine can also block ACTH secretion (7, 8).

In the case of FSH, atropine implants in the hypothalamus but not in the cerebral cortex also blocked the postcastration rise. Since implants into the pituitary of cholesterol alone as well as atropine suppressed the postcastration rise in this gonadotropin, we cannot be sure that the action of atropine to inhibit FSH release was not mediated on the anterior pituitary by uptake of the alkaloid into portal vessels and subsequent delivery to the gland. This is unlikely in view of the failure of pituitary implants of atropine to inhibit the LH rise. Probably the failure of FSH to rise following implantation of cannulae into the pituitary is related to mechanical disruption of anterior pituitary FSH release by the implantation procedure since it was equally pronounced with cholesterol implants.

In conclusion, the present results support earlier studies which suggest that acetylcholine may play a role as a synaptic transmitter to stimulate release of LH-releasing hormone (LHRH) which would in turn increase gonadotropin release. The postulated cholinergic synapses would be located in the anterior and middle hypothalamus.

Summary. Bilateral implants of atropine sulphate were placed in various loci in the brain or into the anterior pituitary in male rats and the effects of the implants on the postcastration rise in plasma FSH and LH was determined. The increase in both gonadotropins at 16 hr after castration still occurred in animals with implants in the

cerebral cortex. The postcastration rise of both FSH and LH was blocked by atropine implants in the anterior, middle, or posterior hypothalamus but was not interfered with by control implants of cholesterol. Bilateral implants of either cholesterol or atropine into the anterior pituitary failed to alter the increase in plasma LH following castration but both types of implants interfered with the postcastration rise in FSH, possibly because of trauma to the pituitary from the cannulae. It is suggested that hypothalamic cholinergic synapses may play a role in stimulating the increased LHRH release which induces the postcastration rise in gonadotropins.

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3S OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **152**, 147-150 (1976)

Nucleation of Microbiologic Calcification by Proteolipid (39348)

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objective of this investigation was to the component of crude phospho- esponsible for *Bacterionema matru-* calcification. The microorganism, an mycete of the human oral biota, forms llular calcium phosphate deposits give diffraction maxima characteristic tite (1). *B. matruchotii* calcification is lependent (2) with activity restricted crude phospholipid (3). A complex ed to be a lipoprotein (4) is present in *truchotii* crude phospholipid. Calcium g by phospholipid-protein complexes en reported (5). The possibility that omplexes participate in vertebrate cal- ion has been considered (6). These in addition to calcification of a syn- illy prepared lipoprotein (7), empha- he probable importance of the pro- ntaining portion of *B. matruchotii* phospholipid.

erials and methods. Crude phospho- was obtained from *B. matruchotii*, 14266. The organism was grown 7 t 37° in 250-ml vol of a chemically d medium (Table 1). Twenty-liter s were harvested by centrifugation 0 *g*, 5 min, 5°). Cells were water d and freeze-dried without protective l. Total lipid was extracted by sus- g the full yield of dried cells, usually 5 g, in 300 ml of chloroform-metha- 1, v/v (CM) for 18-20 hr at room rature. The extract was filtered (Milli- JH) and nonlipids were removed by a l, 0.12 *M* sodium chloride wash (8). hloroform phase was drawn off and ntrated to 1/8 vol under vacuum at 45°. e was restored with acetone and the t held at 5° for 24-36 hr. The crude olipid precipitate was recovered by ugation, acetone-washed, and imme- dissolved in 10 ml of CM.

de phospholipid was fractionated by n chromatography with hydroxypropylated dextran gel (Sephadex LH-20). The CM solution was applied in two 5-ml vol to a 2.5 × 51.0-cm column equilibrated with CM. To detect protein, each 320-ml elution was monitored with 280 nm ultraviolet. Individual fractions and various recombinations were stored at −15° until dried at 45° under nitrogen flow.

Samples selected for calcifiability tests were: (a) all fractions recombined, (b) the single, protein-containing fraction, and (c) all fractions recombined except the protein-containing fraction. Fifteen-milligram quantities of each were incubated at 37° in airtight 50-ml centrifuge tubes filled with carbonate-buffered pH 7.20 metastable calcium phosphate solution (9) containing 0.02% thymol. At 24-hr intervals pH determinations were made, the tubes were centrifuged, and the solutions changed. Incubation periods ranged from 96 to 192 hr. At termination the samples were examined for microbial contamination, water-washed, air-dried, and heated to 700°. Residues were examined by X-ray diffraction using a powder-camera film technique with nickel-filtered copper radiation generated at 30 kV, 20 mA anode current for 5 hr.

The protein-containing fraction was partially characterized. Amino acid composition was determined (Phoenix K-800 Autoanalyzer) from an acid hydrolysate. General phospholipid identification was done with thin-layer chromatography (10).

Results. The fortuitous occurrence of two separated ultraviolet absorbances (Fig. 1) during elution permitted division of the crude phospholipid into five fractions. The solid phase obtained after incubation of either fraction 2 or the recombined five fractions in the metastable calcium phosphate solution, when ashed, gave X-ray diffraction patterns for apatite (Fig. 2). Fraction 2 calcified within 96 hr. The recombined fractions required 168 hr. No evidence for apa-

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served.

TABLE I. MEDIUM FOR *Bacterionema matruchotii.*

	Per Liter
Glucose	2.00 g
Casein hydrolysate, vitamin-free	4.00 g
Salts	
$NaHCO_3$	1.85 g
Na_2HPO_4	550 mg
CH_3COONa	147 mg
$CaCl_2 \cdot 2H_2O$	14.5 mg
$MgSO_4 \cdot 7H_2O$	35 mg
$FeSO_4 \cdot 7H_2O$	4 mg
$MnSO_4 \cdot H_2O$	0.15 mg
$NaMoO_4 \cdot 2H_2O$	0.15 mg
Vitamins	
Para-aminobenzoic acid	2.0 mg
Riboflavin	2.0 mg
Calcium pantothenate	2.0 mg
Inositol	2.0 mg
Thiamine	2.0 mg
Nicotinic acid	1.0 mg
Pyridoxine HCl	1.0 mg
Biotin	0.1 mg
Folic acid	0.1 mg
Growth Factors	
Pimelic acid	0.1 mg
Thioctic acid	0.1 mg

Nitrogen bases: 20 mg of each of adenine, guanine, thymine, uracil, xanthine dissolved in 100 ml of 0.1 *M* KOH.

Medium is made up in 0.15 *M* N-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid adjusted to pH 7.4 with NaOH. Filter-sterilization is required.

tite formation was obtained with the recombined fractions minus fraction 2 exposed to the calcium phosphate solution for 192 hr.

The solutions containing either fraction 2 or recombined fractions gave daily postincubation pH readings between 6.90 and 6.98. With recombined fractions minus fraction 2 the readings were consistently 7.20. At termination none of the samples was contaminated.

Preliminary analyses indicated approximately 38% of fraction 2 was protein. The amino acid data obtained are given in Table 2. The remainder of the fraction consisted of cardiolipin, inositides, and possibly other acidic phospholipids. Dried fraction 2 was a light brown, granular powder. It was insoluble in water and salt solutions and soluble in chloroform–methanol mixtures. Each of the other dried fractions was a lipidlike semisolid with no protein detectable by biuret or ninhydrin.

Discussion. The component of *B. matruchotii* crude phospholipid responsible for calcification is a protein–lipid complex with lipidlike solubilities, a proteolipid. Proteolipids, first noted in brain tissue, are found in a variety of biologic membranes (11). Proteolipid involvement in microbial calcification is additional evidence that initiation of *B. matruchotii* calcification is membrane-associated (12). More important than spatial considerations, the finding is the first instance in which a specific chemical entity has been shown experimentally to be essential for a form of biologic calcification. Further, the proteolipid was isolated from a fastidious organism cultivated in a chemically defined medium. This provides for readily controllable investigation of biosynthesis related to biologic calcification.

The incubation time for fraction 2 calcification is relatively unimportant at this stage of investigation. The calcification procedure used was a closed system which, while preventing contamination and pH elevation, underwent progressive ion depletion between solution changes. Greater calcification efficiency would be shown with a continuous-flow procedure. A more meaningful measure of efficiency, however, would be to determine the minimum calcium × phosphate product required for nucleation.

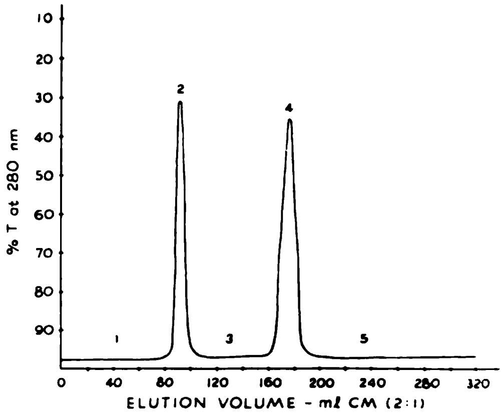

FIG. 1. Hydroxypropylated dextran gel (Sephadex LH-20) column chromatography of *B. matruchotii* crude phospholipid. Five-milliliter aliquots of sample in chloroform methanol 2:1, v/v (CM), were applied to 2.5 × 51-cm column equilibrated with CM. Elution volumes of 4 ml were pooled to yield five fractions.

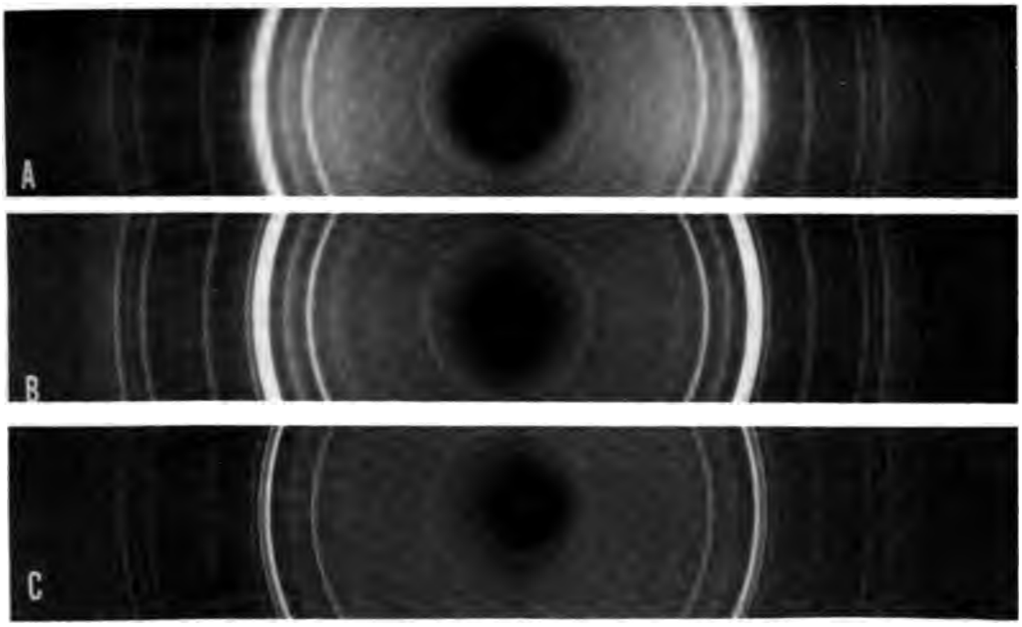

FIG. 2. X-ray diffraction patterns of ashed residues from fraction 2 (A) and recombined five fractions (B) of *B. matruchotii* crude phospholipid after incubation in metastable calcium phosphate solution. A pattern from human dentin (C) is a biologic apatite reference.

TABLE II. AMINO ACID COMPOSITION OF *Bacterionema matruchotii* CRUDE PHOSPHOLIPID FRACTION 2.

Amino acid	Protein (m*M*/mg)
Aspartic acid	0.75
Glutamic acid + proline	0.89
Serine	0.80
Threonine	0.40
Valine	0.14
Methionine	0.19
Tyrosine	0.05
Phenylalanine	0.25
Glycine	1.29
Alanine + cysteine	1.16
Leucine	1.27
Isoleucine	0.43
Lysine	0.55
Histidine	0.11
Arginine	0.68
NH_3	0.80

The preliminary amino acid data do not permit an accurate determination of whether the protein is acidic or basic. Therefore the type of protein-acidic phospholipid interaction cannot be clarified. However, the presence of both hydrophobic and basic amino acids would provide binding sites for acidic phospholipids. Irrespective of the nature of the relationship, the acidic phospholipids, except for cardiolipin, are difficult to dissociate from the protein.

Crude phospholipid is necessary for calcification, *in vitro*, of matrices prepared from bone (13) and elephant tusk (14). The critical issue is whether proteolipid is the active fraction in bone and tusk calcification. If so, *B. matruchotii* can be considered a microbiologic analogue for nucleation of these forms of vertebrate calcification.

Summary. The component of crude phospholipid responsible for *B. matruchotii* calcification was isolated. Crude phospholipid, extracted from the microorganism, was separated into five fractions by column chromatography. A single, protein-containing fraction catalyzed apatite formation in a metastable calcium phosphate solution. The nucleating fraction was identified as a proteolipid.

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Spleen Lymphocyte Bactericidal and Peroxidase Activities: Enhancement by Whole Body X-Irradiation[1] (39349)

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ocytes collected from x-irradiated
s generally indicate depression in
ytosis-associated activities (1-3). In
orts to investigate the effect of x-
ion on host-defense, the principal
ıdied have been the guinea pig peri-
exudate PMN (1-3) and human pe-
l leukocytes (4, 5). For comparative
es, and in order to broaden our un-
ding of various aspects of host-de-
ve have extended our investigation to
the bactericidal and metabolic activ-
spleen cells (predominantly lympho-
ollected from nonirradiated and to-
y x-irradiated mice (6).

ealized that spleen cells, mainly lym-
es, as judged by light microscopy, are
nerally considered as conventional
ytic cells. However, in previous stud-
ıave noted that these cells are able to
teria and, when challenged with par-
hey respond metabolically similar to
tional phagocytic cells (6, 7). Elec-
croscope studies have indicated (un-
ed results) that actual particle entry
ot occur. It would appear that the
lic and bactericidal changes noted
due to conventional phagocytosis
to cell surface-particle interaction.
basis of histochemical data, our pre-
y results indicate that peroxidase is
in these cells. Generally others have
d little or no peroxidase activity in
ells. However, by the guaiacol perox-
say (7), we have observed here that
ouse spleen lymphocytes have signif-
roxidase activity. Many peroxidases
yelo, lacto, horseradish, and macro-
eroxidase) and H_2O_2, in the presence of a halide ion, kill a variety of microorganisms (8-11). Some of the biochemical and bactericidal activities of CD1 mouse spleen lymphocyte peroxidase (SLPO) and H_2O_2 and the effect of whole body x-irradiation and particle addition on these functions are reported here.

Methods and materials. CD1 mice obtained from the Charles River Laboratory, Boston, Massachusetts, and subsequently bred in St. Margaret's Hospital animal quarters were used. Animals of either sex, weighing approximately 20-30 g (8-12 weeks) were x-irradiated in a Portable Andrex X-Ray unit, as previously reported (1-3).

Total and differential white blood cell counts were determined on blood collected from the tail vein immediately before and also generally at 24 hr after x-irradiation. The animals were sacrificed at 1, 3, 5, 10, and 15 days after x-irradiation by cervical dislocation. Spleen cells from nonirradiated mice (8-12 weeks old) served as controls. The spleens were freed from fatty tissues, washed with Krebs-Ringer phosphate medium (KRPM, pH 7.4), and placed on filter paper. The wet weight of each spleen was recorded. Spleen cells were then prepared and counted, as previously reported (12, 13). Total leukocyte and differential counts were noted. The cells were suspended in KRPM for use in metabolic and bactericidal studies.

The oxidation of [1-^{14}C]glucose and [6-^{14}C]glucose was studied in single-arm Warburg flasks. The reaction mixture in the main compartment contained 30 μmol of glucose (sp act 0.01 μCi/μmol of glucose) and 20-25 × 10^6 spleen cells in a total volume of 3 ml of KRPM, pH 7.4. To study the effect of particle addition on glucose oxidation, polysyrene latex spherules of

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erved.

0.81-μm diameter were added at a cell to particle ratio of 1:100. The $^{14}CO_2$ resulting from the oxidation G-1-^{14}C or G-6-^{14}C was counted in a Packard liquid scintillation spectrometer equipped with an external standard. The details on the procedure have been reported earlier (10, 12, 13).

Preparation of peroxidase-containing 20,000 *g* granules and estimation of peroxidase activity: Spleen cells collected from x-irradiated and nonirradiated mice were freed of red blood cells by cold water lysis (7, 12, 13). The cells were then suspended in a desired volume of 0.25 *M* sucrose and homogenized for 5 min in a motor-driven Teflon-tipped Potter–Elvejhem glass homogenizer. The homogenate was centrifuged at 20,000 *g* for 30 min at 4° in a refrigerated Sorvall RC 2 centrifuge. The 20,000 *g* pellet was resuspended in a 0.25 *M* sucrose. The peroxidase activity of the 20,000 *g* granules was estimated by both guaiacol oxidation (at pH 7.0 in the presence of 0.25 *M* sucrose) and decarboxylation activity (KRPM, pH 5.5). Both methods have been previously described (7, 10, 12, 13).

Bactericidal Activity. Both intact cells and 20,000 *g* granules were assayed for bactericidal activity. The test organism, *Escherichia coli*, was grown overnight, harvested in the logarithmic growth phase, washed, and resuspended in KRPM of pH 5.5. The Reaction mixture contained 2 × 10^4 to 2 × 10^8 *E. coli* and the following supplements: Granules equivalent to 0.003–0.03 guaiacol unit, H_2O_2, 0.1 μmol, and KRPM, pH 5.5, to a final volume of 2.0 ml. (For assaying intact spleen cells, 5 × 10^6 spleen cells and 5 × 10^6 *E. coli* were incubated for 60 min at 37° in KRPM of pH 7.4.) After a 30-min incubation period at 37°, aliquots of the reaction mixture were plated in trypticase soy agar and colonies counted at 24 hr (10, 12, 13).

All chemicals were of reagent grade. [1-^{14}C]Glucose, [6-^{14}C]glucose, and L-[1-^{14}C]alanine were obtained from the New England Nuclear Corporation, Boston, Massachusetts. Polystyrene latex particles of 0.81-μm diameter were obtained from the Difco Laboratories, Detroit, Michigan.

Results. The peroxidase activity of the 20,000 *g* granules isolated from mouse spleen cells collected 24 hr after whole body x-irradiation is presented in Table I. It can be seen that on a per cell basis, the guaiacol oxidation by granules from 400 R x-irradiated mice spleen is approximately fivefold more than that of control. This guaiacol oxidation activity, assayed in the presence of 0.25 *M* sucrose is approximately 40% of that in the absence of sucrose. However, sucrose has been used in order to protect the peroxidase-containing granules from lysis. The decarboxylation of L-[1-^{14}C]alanine by this fraction is also presented in Table I. This reaction also measures peroxidative activity. A dramatic increase in $^{14}CO_2$ formation by granules collected from x-irradiated mice is noted when compared to the decarboxylation activities of the corresponding nonirradiated controls.

The 20,000 *g* granules isolated from spleen cells of x-irradiated and non-irradiated mice were tested for their bactericidal activity (Table I). The granules collected from mouse spleens 1 day after 400 R x-irradiation had significantly higher bactericidal activity when compared to spleen cell granules collected from nonirradiated controls.

Intact spleen cells collected 1 day after 400 R whole body x-irradiation (at a cell to bacterium ratio of 1:1) killed approximately 2 logarithms of *E. coli* as compared to approximately 1 log by spleen cells from nonirradiated controls.

TABLE I. PEROXIDASE AND BACTERICIDAL ACTIVITIES OF THE 20,000 *g* PELLET ISOLATED FROM SPLEEN CELLS OF NON AND X-IRRADIATED MICE.

	Control	X-irradiated[a]
Guaiacol oxidation[b]	0.15	0.69
Decarboxylation[c]	78.3	2193
Bactericidal activity[d]	2–5	4–7

[a] Results (three or more experiments) of cells collected 1-day post 400 R x-irradiation are presented. The activities of preparations from x-irradiated mice are significantly higher than those from control mice.

[b] Guaiacol unit per 10^8 cells. The assay procedure has been published (7, 10, 12, 13).

[c] Nanomoles of L-[1-^{14}C]alanine decarboxylated to $^{14}CO_2$ in 30 min at 37° by 20,000 *g* granules containing 0.03 guaiacol unit of peroxidase.

[d] Logarithms of *E. coli* killed by freshly prepared 20,000 *g* granules containing 0.003–0.03 guaiacol unit of peroxidase in 30 min at 37°.

The amount of $^{14}CO_2$ formation from the oxidation of [1-^{14}C]glucose is an approximate measure of the amount of glucose metabolized by the hexose monophosphate shunt (HMS) pathway. One hundred million spleen cells collected from nonirradiated mice oxidized 27 nmol of glucose through the HMS in 30 min at 37°. The addition of polystyrene latex particles resulted in a fourfold increase in HMS activity. Spleen cells, in the absence of added particles, collected 1 day after 400 R x-irradiation oxidized 69 nmol of [1-^{14}C]glucose and this result is significantly higher than that of nonirradiated controls. If polystyrene latex particles are added to cells collected from x-irradiated mice, 240 nmol of [1-^{14}C]glucose are oxidized. These results can be seen in Table II. The amount of $^{14}CO_2$ oxidized from [1-^{14}C]glucose due to particle addition to spleen cells from nonirradiated as well as 100 to 800 R x-irradiated mice are always significantly higher than the corresponding $^{14}CO_2$ formed in the absence of particles. The extent of oxidation of [6-^{14}C]glucose is less than that of [1-^{14}C]glucose. X-irradiation and particle addition have a stimulatory effect though the overall oxidation is less than that of [1-^{14}C]glucose. These results also can be seen in Table II.

The effects of various doses of whole body x-irradiation on peripheral white blood and spleen cells are presented in Table III. It can be seen that with increasing doses of x-irradiation, the weight of the spleen, the total number of spleen cells and the total white blood cells of the peripheral circulation are decreased significantly in 24 hr. A correlation appears to exist between the doses and the changes in these parameters within 1 day after x-irradiation. In general, in the peripheral blood and spleen, the percentage of lymphocytes decreases while the percentage of segmented cells increases with increasing x-irradiation doses.

The results presented in Tables I and II are from 1 day post 400 R x-irradiation. Effects of 100, 200, or 800 R have also been investigated and are, in general, proportional to the increasing doses of x-irradiation. The extent of stimulation of these activities (glucose, alanine and guaiacol oxidations, antimicrobial and cell differentials) generally increases with increasing days post x-irradiation up to 5 days. Ten to fifteen days post 100 or 200 R x-irradiation all of

TABLE II. EFFECT OF WHOLE BODY X-IRRADIATION (400 R) AND PARTICLE ADDITION ON THE OXIDATION OF GLUCOSE BY MOUSE SPLEEN CELLS.

	Control		X-irradiated	
	Particles		Particles	
	−	+	−	+
G-1-^{14}C	27[b]	103	69	240
G-6-^{14}C	6[c]	15	16	76

[a] The ratio of spleen cell: Polystyrene latex particles (0.81 μm diameter) was 1:100.

Please see text and Table I for additional details.

[b] Nanomoles of $^{14}CO_2$ oxidized from [1-^{14}C]glucose by 10^8 cells in 30 min at 37°.

[c] Nanomoles of $^{14}CO_2$ oxidized from [6-^{14}C]glucose by 10^8 cells in 30 min at 37°.

TABLE III. EFFECT OF WHOLE BODY X-IRRADIATION ON MOUSE SPLEEN AND PERIPHERAL BLOOD LEUKOCYTES.[a]

	Spleen wet weight (mg)	Total spleen cells × 10^6	Total WBC peripheral × 10^6	Cell differentials (%)[b]			
				Peripheral		Spleen Cells	
				L	S	L	S
Nonirradiated	134	62	16	66	27	95	4
1-day post x-irradiation and dose (R)							
100	101	43	12	60	35	84	15
200	75	28	5	42	54	73	21
400	58	14	6	28	66	65	34
800	54	7	4	17	78	66	34

[a] Average from 10 or more mice is presented.

[b] L, lymphocytes; S, segmented cells. Young immature myelocytic cells, monocytes, and eosinophils are not tabulated but never amount to greater than 1% in spleen except in 200 R.

these activities generally returned to normal levels.

Discussion. It is apparent from the results of this study that whole body x-irradiation stimulates the *in vitro* HMS, peroxidative and bactericidal activities of mouse spleen cells. Increased HMS could supply some of the H_2O_2 required for the stimulated decarboxylation and bactericidal activities.

Recently it has been reported that the *in vivo* survival of ***Brucella abortus*** in the spleens of previously irradiated (700 R) CBA mice is significantly decreased as compared to normal (14). A possible explanation for this observation is our present finding of increased peroxidase activity in spleen lymphocytes from x-irradiated animals.

In attempting to explain the stimulated activities following *in vivo* x-irradiation, one must consider the significant change in lymphocyte/phagocyte ratio in the spleen. The percentage of lymphocytes has decreased while the percentage of polymorphonuclears has increased in both the spleen and the peripheral blood (Table III). Although myeloperoxidase of PMN has been reported to be approximately three times more potent on equal guaiacol unit bases than spleen cell peroxidase (SPO) from AKR mice (12, 13), the dramatic stimulations and increased bactericidal activities noted could not be fully accounted for by the percentage decrease in lymphocytes and a simultaneous percentage increase of PMN. For example, an MPO preparation from guinea pig peritoneal cells (over 90% PMN) at concentrations (i.e., 0.003–0.03 guaiacol unit in 2-ml reaction medium) identical to that used in this study killed approximately 1×10^3–1×10^5 bacteria. In this present study the granular (SLPO) preparation from the spleen cells of x-irradiated mice killed approximately 1×10^4–1×10^7 bacteria (Table I). The increased percentage of phagocytes in x-irradiated spleens may account for some of the increase in activities. However, lymphocytes are in the majority, and it seems reasonable to assume that lymphocyte peroxidase plays a major role in this increased bactericidal activity.

To examine further whether the increased percentage of granulocytes from x-irradiated mice spleen could contribute to the stimulated activity, we have attempted to remove the granulocytes from spleen cell preparations by allowing them to adhere to Falcon plastic tissue culture flasks. Complete removal of granulocytes, especially from x-irradiated mice spleen, has not yet been achieved. However, removal of 50% of the granulocytes from an irradiated spleen cell population had no effect on the bactericidal activity of the 20,000 *g* fraction. This occurred with different concentrations of peroxidase. For example, with 0.003 guaiacol units approximately 10^5 bacteria out of a total of 10^8 bacteria are killed. Removal of 50% PMN from this preparation and then testing 0.003 units, as previously reported, resulted in identical killing. Therefore, the major factor in the dramatic increase in decarboxylation and bactericidal functions by peroxidase from x-irradiated spleen cells must be attributed to enhanced activity of the lymphocytic peroxidase.

The decarboxylation and bactericidal activities of the 20,000 *g* granules collected from non- and x-irradiated mouse spleen may be dependent on the rate of peroxidase-substrate interaction. For example, we have observed that freshly prepared granules from x-irradiated mice have significantly higher bactericidal activity than that of nonirradiated controls. If the preparation from nonirradiated control is frozen and thawed, the bactericidal potency of this preparation is increased. It appears that release of peroxidase from the granules is necessary for optimal utilization of the enzyme. The stress of *in vivo* x-irradiation may in general be compared to the stress of addition of phagocytizable particles. In the latter event, lysosomal enzymes are released from the granules to the phagocytic vacuoles (15). This stress could favor the utilization of the substrates by the released enzymes. It may be mentioned here that the viability (as judged by eosin dye exclusion) of spleen cells 1 day after 400 R x-irradiation is approximately 50% as compared to that of 85–90% for spleen cells from nonirradiated controls. X-irradiation perhaps alters cellular permeability and, therefore, favors the interaction of the peroxidase and its substrates (H_2O_2, guaiacol, bacteria, etc.) and this may account for the enhanced activities observed. It appears that the spleen cell enzyme is made easily available to the substrates (H_2O_2, amino acids, chloride, bacteria, etc.) by x-irradiation, by lysis (freezing and thawing), or by isolation and purification. It is pertinent to mention here our observation that the bactericidal activity of a peroxidase isolated and partially purified from normal mouse spleen cell is equivalent to that of 20,000 *g* granules from x-irradiated mouse spleen (0.03 guaiacol unit of the partially purified peroxidase kills 10^7 *E. coli* in 30 min at 37°) (16).

Optimal activity of SLPO, as determined by guaiacol oxidation, occurs at a pH different than that routinely used for MPO. Concentration of substrates (H_2O_2 and guaiacol) required for SLPO is also different than those used for MPO. However, both SLPO and MPO have strong affinities for H_2O_2 and chloride. Both chlorinate and decarboxylate amino acid and both enzymes can participate in peroxidase-mediated bactericidal activities (6, 8, 10). Recently, we have isolated a peroxidase from a preparation of human peripheral blood containing 100% lymphocytes. These results will be reported in a later publication.

Our results indicate that a lymphocyte peroxidase, not MPO, is responsible for the antimicrobial and biochemical activities of spleen cell preparations. It would be of interest to investigate whether the lymphocyte enzyme is organ-specific or species-specific or universally present in all lymphocytic cells and to determine its role in lymphocyte function.

Summary. A 20,000 *g* pellet obtained from the homogenate of CD1 mouse spleen cells has measurable peroxidase and bactericidal activities. Whole body x-irradiation stimulates the *in vitro* peroxidative and bactericidal activities of the spleen cells. These cells do not phagocytize. However, addition of polystyrene latex particles to spleen cells from non- or x-irradiated mice, increases glucose oxidation. The extent of this stimulation (three- to fourfold) is less than that of PMN (six- to tenfold). Interaction between particles and lymphocyte surface may be the cause of this stimulation. The increased bactericidal activity due to x-ray is attributed mainly to an increase in the peroxidase activity of the lymphocytes. Surface (membrane) action rather than phagocytosis may be involved in the bactericidal process. The spleen lymphocyte peroxidase is distinct from myeloperoxidase in several respects.

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Dose-Response Hyperplasia and Neoplasia from Feeding *N*-2-Fluorenylacetamide (2-FAA) to BALB/c Mice for Varying Time Intervals (39350)

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A dose-response-related urinary bladder hyperplasia in both sexes of BALB/c strain mice fed *N*-2-fluorenylacetamide at dietary concentrations ranging from 10 to 500 ppm for 90 days was reported by Haley *et al.* (1). After consuming 500 ppm of the carcinogen for 90 days, one male mouse developed urinary bladder carcinoma. Another male mouse receiving 250 ppm also developed a similar carcinoma. Clayson *et al.* (2) also observed bladder hyperplasia after feeding the carcinogen at concentrations of 300 ppm for 2 weeks. They suggested that there is a reasonable correlation between early hyperplasia and the final development of carcinoma. However, feeding *N*-2-fluorenylacetamide, 100 ppm, for 455 days resulted in only 3 of 33 mice showing bladder hyperplasia and no carcinoma (Clayson and Bonser, 3). Moreover, 1-month treatment with the chemical resulted in bladder hyperplasia which was present when the experiment was concluded. The males had a greater response than the females. Haley *et al.* (4) obtained a dose-response related urinary bladder hyperplasia in BALB/c mice fed *N*-2-fluorenylacetamide concentrations varying from 8 to 86 ppm for 3 months. Their males were also more responsive than their females. No carcinomas were produced in that experiment. In contrast to Clayson and Bonser (3), no lethality from the chemical was observed. Upon the basis of the above reports, two questions arise: (i) Is the presence of *N*-2-fluorenylacetamide required to prolong urinary bladder hyperplasia once it is induced? (ii) Will continuous feeding of the chemical for 12 months convert the hyperplastic state into carcinoma and at what time interval? We have investigated these questions with large numbers of BALB/c mice and a serial sacrifice design.

Methods. BALB/c strain mice, 3360, equally divided between the sexes were used. The animals were maintained under minimum disease conditions in quarters thermostatically regulated to 24° with free access to food and water. The mice weighed from 20 to 30 g. The sexes were randomized as to cages and racks. The mice were divided into two experiments of 1680 mice each; one group received the carcinogen for 3 months, then were fed control diet for the ensuing 9 months, while the second group received the carcinogen for 12 months. In each experiment the groups were composed of 240, 480, 480, 240, 160, and 80 animals, equally divided by sex and fed dietary concentrations of *N*-2-fluorenylacetamide of 0, 8, 24, 45, 59, and 86 ppm, respectively. *N*-2-fluorenylacetamide dietary content was determined by the methods of Bowman and King (5). Sacrifice periods were 3, 5, 7, 9, and 12 months, and 1/5 of the animals were sacrificed at each time interval. All mice were necropsied and the following tissues were examined microscopically after staining with hematoxylin-eosin: lungs, heart, aorta, thymus, anterior femoral muscle, kidneys, adrenal, liver, spleen, gallbladder, pancreas, cerebellum, spinal cord, stomach, colon, ileum, duodenum, cervical lymph node, salivary glands, lacrimal gland, eye, harderian gland, thyroid, trachea, esophagus, skin, tongue, sternum, testes, epididymus, preputial gland, seminal vesicle, coagulating gland, ovary, uterus, mammary gland, urinary bladder, prostate, pituitary, and any abnormal tissue masses. All data, including physical condition, were stored in a Modular Computer Systems III/5 minicomputer, and the histopathological information was coded on mark sensitive forms and entered into the system with an optical reader. All animals were weighed weekly as individuals on an electronic balance interfaced with a minicomputer.

To determine the possible activation or

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served.

ıction of *N*-2-fluorenylacetamide with
ıe leukemic viruses, assays for Sendai,
ıma, and murine leukemia virus
.v) were conducted at 5-, 7-, 9-, and
onth sacrifice intervals. Severs (6) and
:r *et al.* (7) methods were used for
ıma and Sendai viruses and the Kle-
et al. (8) and the Rowe *et al.* (9)
ods for MuLv.

ults. General observations. The previ-
observed calcareous pericarditis (4)
observed in the controls and treated
als with the condition more pro-
ced in the males than in the females.
: were no significant effects on body
ıt in any group in either experiment.
thosis, ulceration, and acute and
ıic inflammation of the skin was ob-
d only in the males in both experiments
vas probably related to aggressiveness.
: I lists the toxic effects (pooled data)
ıced by feeding *N*-2-fluorenylacetam-
ver 12 months with sacrifices at 3-, 5-,
, and 12-month intervals, and it can be
that the changes were more pro-
ced in the males. Similar changes were
ned in the experiment where the chem-
ras fed for only 3 months and the liver
ges suggest that permanent damage had
dy occurred at this time. The liver
ges confirm (Clayson and Bonser (3).
th experiments, no significant changes
observed in the aorta, thymus, ante-
femoral muscle, kidney, adrenal,
n, pancreas, cerebellum, spinal cord,
ıch, colon, ileum, duodenum, cervical
ı nodes, eye, thyroid, trachea, esophagus, tongue, sternum, testes, epididymus, preputial gland, seminal vesicle, coagulating gland, ovary, uterus, mammary gland, prostate, or pituitary.

Urinary bladder hyperplasia and neoplasia. When 2-FAA was removed from the diet after feeding for 3 months, the hyperplasia in males decreased to almost zero (Fig. 1). The females at 59 and 86 ppm showed hyperplasia in 10/15 and 8/8 mice at 9 months and an increase at 12 months. No carcinoma was observed. However, when 2-FAA was fed for 12 months a dose-related hyperplasia occurred in the males. Of importance is the appearance of hyperplasia in the lowest dose group of the males (Fig. 1). Thus it appears that total dose or continual dosage, not the rate of delivery, is the important factor in hyperplasia development. A pronounced difference in response in females is also evident and it is probably related to the degree of metabolic conversion of 2-FAA by this sex (15). The relationship of dose to urinary bladder carcinoma in males is shown in Table II. No urinary bladder carcinoma was found in the females. Apparently a pronounced and fully developed hyperplasia is essential for the development of neoplasia. The above results do not appear to be related to activation of oncogenic viruses by 2-FAA, or dose of 2-FAA to the proportion of mice with positive virus counts, or to the proportion of virus positive mice at each stage of hyperplasia or neoplasia when analyzed statistically. However, virus counts do increase with age.

Other neoplastic changes. Table III lists

3LE I. TOXIC EFFECTS FROM FEEDING *N*-2-FLUORENYLACETAMIDE TO BALB/c MICE (POOLED SACRIFICE DATA).

	Fed only 3 months				Fed up to 12 months			
	Control		Treated		Control		Treated	
:ffect	♂	♀	♂	♀	♂	♀	♂	♀
fatty meta-osis	8/111*	0/115	45/674	2/707	8/109	0/119	54/677	0/696
fibrosis	2/111	0/115	16/674	2/707	0/109	0/119	15/677	0/696
necrosis	6/111	2/115	30/674	2/707	3/109	0/119	31/677	7/696
acute in-ation	8/111	3/115	30/674	8/707	2/109	2/119	31/677	8/696
chronic in-ation	8/111	2/115	37/674	5/707	3/109	0/119	43/677	12/696
nodular lasia	4/111	0/115	17/674	1/707	2/109	0/119	18/677	0/696

umber responding over total number in group.

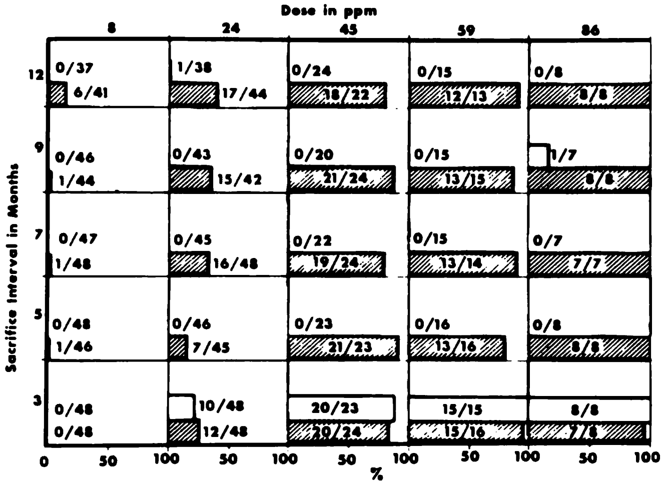

FIG. 1. Bladder hyperplasia in male BALB/c mice fed 2-FAA. Clear area = fed only 3 months; shaded area = fed until sacrificed.

TABLE II. INCIDENCE OF URINARY BLADDER NEOPLASIA IN BALB/c STRAIN MALE MICE FED *N*-2-FLUORENYLACETAMIDE FOR VARYING INTERVALS.

Sacrifice interval (months)	Dose of *N*-2-fluorenylacetamide (ppm)					
	0	8	24	45	59	86
3	0/23*	0/48	0/48	0/24	0/16	0/8
5	0/23	0/46	0/44	0/22	0/16	0/8
7	0/20	0/47	0/47	0/24	0/14	0/7
9	0/20	0/44	0/42	0/24	0/15	3/8
12	0/23	0/41	0/44	1/22	3/13	4/8

* Number responding out of total number in group.

TABLE III. TOTAL NEOPLASIA OCCURRING IN OTHER TISSUES OF BALB/c MICE FED *N*-2-FLUORENYLACETAMIDE (POOLED SACRIFICE DATA).

	Fed only 3 months				Fed up to 12 months			
	Control		Treated		Control		Treated	
Neoplasia	♂	♀	♂	♀	♂	♀	♂	♀
Lung alveolar cell adenoma	10/112*	1/115	52/689	26/710	11/109	7/120	41/677	32/695
Hepatocellular carcinoma	2/110	0/115	5/675	0/709	0/109	0/119	7/677	0/700
Parotid gland adenoma	1/109	0/115	14/662	1/682	1/106	0/116	14/653	3/685
Lacrimal gland adenoma	1/90	2/86	19/596	10/495	5/75	0/67	17/477	3/373
Harderian gland adenoma	1/110	0/115	10/671	11/710	4/109	8/120**	8/677	10/696**
Submaxillary gland adenoma	4/109	0/113	6/671	2/701	3/106	1/116	11/672	3/683

* Number responding over total number in group.

** Significant $\chi^2 = 10.67$; $P = 0.01$; all others not significant.

the other neoplasias produced by feeding 2-FAA for 3 and 12 months. Again a pronounced sex difference is evident, with the males being more susceptible in most cases. There was no significant difference between the treated and the controls.

Discussion. The results obtained by feeding *N*-2-fluorenylacetamide for periods of 3 or 12 months indicate that the presence of the chemical is essential for sustaining urinary bladder hyperplasia and possibly causing the induction of neoplasia of this organ. These data confirm the correlation between bladder hyperplasia and neoplasia suggested by Clayson *et al.* (2), and mammary gland hyperplasia and neoplasia reported by Fisher *et al.* (10). The present results, contrary to those of Clayson and Bonser (3), d that both bladder hyperplasia and asia can be produced by feeding 2- at lower doses for shorter time pe- Moreover, chemical lethality did not , thus confirming the results of Levi *et* 1) and Haley *et al.* (1), but not those ed by others (12–14). Our observa- regarding the differences in response, hyperplasia and neoplasia related to upport the report of Armstrong and r (15) regarding differences in re- e related to both strain and sex of the used.

mary. Hyperplasia induced by *N*-2- nylacetamide requires the continuous ce of the chemical for its mainte- . Feeding the chemical at levels of 8 to m results in a dose-response related y bladder hyperplasia and, after 9 to onths, in bladder neoplasia at the dose levels. Neoplasia of the lungs, parotid gland, lacrimal gland, harder- and, and submaxillary gland was also ved. Measurement of oncogenic vi- revealed no synergism with 2-FAA o relationship between virus counts eoplasia. Calcareous pericarditis was ed and most general toxicological ef- ccurred in the liver. A pronounced ifference in response was observed e males giving greater responses than nales in all instances.

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Polyglutamic and Polyaspartic Acids: Synthetic Polypeptides with Predominantly Factor VIII-like Coagulant Activity[1] (39351)

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Several years ago we reported that proteins of small molecular weight with specific F. (factor) VIII-like activity are present in purified preparations of leucineaminopeptidase made from porcine and canine kidneys (1, 2). Later, we demonstrated that treatment with succinic anhydride of human plasma (3) or purified human albumin (4) produces a F. VIII-like activity of small molecular size. Since succinic anhydride irreversibly attaches carboxyl groups to exposed epsilon-amino groups of proteins, it occurred to us that the F. VIII-like activity of the succinylated proteins might be an expression of the increased number of carboxyl groups. If this idea were correct, then synthetic polyamino acids rich in carboxyl groups, such as polyglutamic and polyaspartic, should also have F. VIII coagulant activity. This paper describes our experiments with these synthetic polypeptides which do indeed show considerable F. VIII activity *ex vivo*. Quite unexpectedly they also have weak F. IX activity. Some of these findings were reported earlier (5).

Materials and methods. Poly-L-glutamic, poly-L-aspartic, and poly-D-glutamic acids of varying molecular weights were purchased from Schwartz-Mann and the Nutritional Biochemicals Corporation. Stock solutions of 1 mg/ml were prepared in distilled water and diluted, before testing, with 0.05 *M* Tris-HCl buffer, pH 6.8, containing 0.15 *M* NaCl. The partial thromboplastin used in our experiments was a crude phospholipid preparation (Thrombofax) distributed by Ortho Diagnostics. Bio-Gel A-15 M, 4% agarose, 200-400 mesh, was obtained from Bio-Rad Laboratories and used in a 2.5 × 45-cm Pharmacia column for separation of factor VIII from Hemophil, the partially purified plasma concentrate made by Hyland Laboratories. Citrated plasmas were collected and specific 1-stage assays were performed for coagulation factors V, VII, VIII, IX, X, and XII (using either the PT or PTT methods) by techniques described previously (1).

The transfusion studies were performed on dogs with the Chapel Hill strain of hemophilia A (6).

Results. Effect of the polypeptides in clotting factor assays. Polyglutamic acids of three different molecular weights, and a single sample of polyaspartic acid, were tested in clotting factor assay systems using plasmas deficient in specific factors. A representative experiment illustrating the effects of the polypeptides on the clotting times in seven different assay systems is shown in Table I. It is clear that none of the polypeptides shortened the clotting times of the F. V, VII, X, or XII deficient plasmas, and there was only a trace of activity in the F. IX assay. However, each of the peptides markedly shortened the clotting time of hemophilic plasma. In addition, polyaspartic acid was as effective against a F. VIII-deficient substrate from a subject with vWd as from a man with hemophilia A.

Specific requirements of kaolin-activation and phospholipid. Table II demonstrates that both kaolin activation of the hemophilic plasma and additional phospholipid are required for expression of the activity of the polypeptides. Hemophilic plasma was mixed with an equal part of buffer, polyglutamic acid (PGA), or normal plasma. One-tenth milliliter of the mixture was added to 0.1 ml of thrombofax and 0.1 ml of $CaCl_2$. Buffer was substituted for the phospholipid in columns 2 and 4. Hemophilic plasma in the first two columns had been activated for 10 min with kaolin before being tested. In the first column (kaolin-activated plasma and lipid present) PGA is almost as effective as normal plasma in correcting the pro-

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TABLE I. Effect of Polyglutamic and Polyaspartic Acids on Clotting Factor Assays: Specific Single Factor-Deficient Plasmas.[a]

					VIII		
	V	X	XII	IX	HEM. A.	vWd	VII[b]
Test material	Clotting times, seconds/units of factor per ml						
Buffer	>250/0	228/0	>260/0	186/0	167/0	183/0	58/0
% Normal plasma	218/.01	167/.01	256/.01	147/.01	139/.01	123/.01	36/.01
0% Normal plasma	122/.20	111/.20	163/.20	103/.20	99/.20	97/.20	17/.20
Poly-L-glutamic acid[c]							
4100 mol wt	>250/0	>230/0	>260/0	132/.06	97/.46	–	75/0
5000–15,000 mol wt	>250/0	216/<.01	>260/0	151/<.01	101/.32	–	78/0
75,000 mol wt	>250/0	>230/0	>260/0	144/.02	106/.24	–	85/0
Poly-L-aspartic acid[c]							
2500–6000 mol wt	>250/0	225/<.01	>260/0	148/.01	95/.52	92/.60	68/0

[a] Plasma obtained from individuals with hereditary, congenital factor deficiencies.

[b] Prothrombin-time assay. All other assays are of the PTT type, kaolin-activated. Each clotting time is the verage of three determinations.

[c] Stock solutions of 1.0 mg of polypeptide/ml buffer were diluted from 20 to 1% and tested in the assays. These lotting times are of the 10% dilutions in each assay.

TABLE II. Necessity of Kaolin-Activation and Phospholipid for Demonstrating the F. VIII-Like Activity of Polyglutamic Acid on Recalcification Times.

	Clotting times, seconds			
Test material	Kaolin and thrombofax	Kaolin, no thrombofax	No kaolin, thrombofax	No kaolin, no thrombofax
Hemophilic plasma + buffer[a]	>250	>250	>250	>250
Hemophilic plasma + PGA[b]	101	>250	>250	>250
Hemophilic plasma + normal plasma[c]	89	172	107	>250

[a] Tris–HCl, 0.05 *M*, NaCl, 0.15 *M*, pH 6.8.

[b] Poly-L-glutamic acid, 175,000 mol wt, 0.1 mg/ml uffer.

[c] Undiluted.

TABLE III.

A. Effect of Poly-L-Glutamic Acid (PGA) of 175,000 mol wt on Partial Thromboplastin Time (PTT) of Plasmas Previously Activated with Kaolin

	Clotting times, seconds	
Type of plasma	Plasma + buffer[a]	Plasma + PGA[b]
Normal	63	59
Factor XII deficient	394	336
Factor X deficient	184	182
Factor IX deficient	260	118
Factor VIII deficient	204	90

B. Effect of PGA of 175,000 mol wt on the PTT of Plasmas Subsequently Activated with Kaolin

	Clotting times, seconds	
Type of plasma	Plasma + buffer[a]	Plasma + PGA[b]
Normal	62	68
Factor XII deficient	>400	>400
Factor X deficient	253	220
Factor IX deficient	241	252
Factor VIII deficient	221	213

[a] Tris-HCl, 0.05 *M*, pH 6.8.

[b] 0.1 mg/ml.

longed clotting time of the hemophilic plasma. Omission of the lipid alone (column 2) or the kaolin alone (column 3) abolishes the F. VIII corrective effect of PGA.

The previous experiment showed that PGA is effective in shortening the clotting time of F. VIII deficient plasmas in the presence of phospholipid when the hemophilic substrate plasma has been activated with kaolin. Table III shows that kaolin activation must occur before addition of the polypeptide. The bottom line of Table IIIA hows the corrective effect of PGA in prioractivated plasma. The bottom line of Table IIB shows the absence of a corrective effect n subsequently activated plasma. (In each instance, the plasma was activated with kaolin for 10 min at 28° before addition of thrombofax and $CaCl_2$.)

The factor VIII "potentiating effect" of polyglutamic acid. When either polyglutamic or polyaspartic acid is added to normal plasma or to fractions of normal plasma containing F. VIII, more F. VIII activity is observed than expected. (The same effect has been seen when kidney AHF or the small-molecular AHF_3 from succinylated albumin had been added to normal plasma and the mixture assayed for F. VIII). We referred to this effect as "potentiation" of plasma F. VIII (2). Table IV shows the degree of "potentiation" of plasma F. VIII when a sample of polyglutamic acid of 75,000 mol wt is added to normal plasma, to Fraction 1-0 (7) and to a highly purified F. VIII preparation. The table also shows the converse, i.e., the absence of "potentiation" when PGA is added to a hemophilic fraction. PGA enhanced the F. VIII activity of each test material that contained F. VIII activity derived from normal plasma by from two- to fivefold while the mixture with hemophilic I-0 was inhibitory. When the mixture of the purified F. VIII fraction and PGA was tested in assays based upon plasma defective in factors other than F. VIII, it lacked potentiating effect. Thus it is seen that the PGA specifically "potentiates" F. VIII.

Behavior of poly-L-glutamic acid in dogs, in vivo and ex vivo. Since polyglutamic acid is very active in F. VIII assays when kaolin-activated human hemophilic plasma is used as substrate, its effect was studied in F. VIII assays based on canine plasma. A variety of polyglutamic and polyaspartic acids of different molecular weights and a wide range of concentrations were studied in F. VIII assays using kaolin-activated plasmas obtained from hemophilic dogs of the Chapel Hill strain (6). No F. VIII-like activity was demonstrated under any of these conditions.

TABLE IV. "POTENTIATION" OF F. VIII ACTIVITY BY PGA.

	F. VIII, u/ml		
Test mixture	Obs.	Exp.	Ratio obs./exp
(1) PGA[a] + buffer[b]	0.38		
(2) Normal plasma + buffer	0.56		
Normal plasma + PGA	4.65	0.94 (1 + 2)	4.9
(3) Normal fraction I-O + buffer	1.35		
Normal fraction I-O + PGA	4.40	1.73 (1 + 3)	2.5
(4) F. VIII fraction [c] + buffer	0.49		
F. VIII fraction + PGA	4.15	0.87 (1 + 4)	4.8
(5) Hemophilic fraction I-O + buffer	<.01		
Hemophilic fraction I-O + PGA	0.20	0.38 (1 + 5)	0.53

[a] Polyglutamic acid, mol wt 75,000, 0.1 mg/ml buffer.

[b] Tris-HCl, 0.05 *M*, NaCl, 0.15 *M*, pH 6.8.

[c] V_0 fraction of "Hemophil" filtered through Bio-Gel A-15 M.

Despite these negative *ex vivo* results, a few *in vivo* experiments were performed. In one experiment, a mixture of 100 mg of poly-L-glutamic acid of 75,000 mol wt, 500 mg of canine albumin, and 2.0 ml of canine brain cephalin (1.5%), in a total volume of 10 ml of 0.15 *M* NaCl were injected into a 9.5-kg hemophilic dog. Blood was drawn at intervals over a 24-hr period following infusion and the following tests were performed: whole blood clotting time, thrombin clotting time, partial thromboplastin time, euglobulin lysis time, and assays for factors VIII, IX, and X. No significant change was observed in any of the tests. Negative results were also obtained when only polyglutamic acid was infused. These *in vivo* results are similar to those previously obtained on infusion into dogs of F. VIII activity of small mol wt isolated from canine kidneys or produced by succinylation of canine albumin (unpublished results). It should be noted, however, that both of these latter preparations had shown factor VIII activity *ex vivo* in canine assays.

Summary and discussion. We have shown that polyglutamic and polyaspartic acids are quite active in specific assays for F. VIII, much less so in specific assays for F. IX, and inactive in four other systems (Table I). The F. VIII and IX activities are demonstrable only when the deficient substrate plasmas have been activated previously with kaolin and when phospholipid is also present (Tables II and III). Furthermore, polyglutamic acid "potentiates" F. VIII activity, two- to fivefold, when it is added to normal human plasma or F. VIII-rich fractions of normal human plasma (Table IV).

"Potentiation" of F. VIII by PGA is apparently different from activation or en-

nent of F. VIII activity by thrombin.
low concentrations of thrombin are
ı to activate F. VIII in a time-depend-
action in which maximum activation
ıot occur until several minutes after
ȝ (8). This increase in F. VIII activity
table, decreasing to the original value
further incubation. In contrast, the
ntiating" effect upon plasma F. VIII
lyglutamic acids (as well as kidney
and AHF$_3$ prepared from albumin) is
/ed immediately upon mixing and re-
stable for hours at room temperature
blished observations).

experiments suggest that polyglu-
acid does not decrease clotting times
tivating factors XII, XI, or IX, but
ınce these factors have been activated
ılin, polyglutamic acid is able to "sub-
" for F. VIII. The fact that the PGA
a slight corrective effect in previously
ted F. IX deficient plasma suggests a
ly even wider role, i.e., that polyglu-
acid may mimic the "intrinsic F. X
tor."

ılin-activation of the F. VIII deficient
ate is an absolute requirement for
sion of the activity of the polypep-
In this respect the F. VIII-like activity
synthetic polypeptides is similar to the
molecular F. VIII activity isolated
cidneys (1, 2) or obtained by succinyl-
of plasma or albumin (3, 4). This pe-
ity suggests that F. IX$_a$ may be re-
d in the test system if the F. VIII activ-
the polypeptides is to be detected.
holipid is also required for the meas-
ent of the F. VIII-like activity of the
eptides, indicating that they are not
mboplastic" in nature.

yglutamic acids active in human F.
ssays were found not to be active in F.
assays based upon hemophilic dog
a, even when kaolin and phospholipid
present, nor to have any effect when
used into hemophilic dogs. "Kidney
" and "albumin AHF$_3$", by contrast
tive *ex vivo* in canine assays, although
oo appear to have no effect *in vivo*.

re may be several explanations for the
f an *in vivo* effect. Polyglutamic acid,
y "AHF" and albumin "AHF$_3$" may
mpletely unrelated to the coagulation moiety recognized as plasma F. VIII. However, if it is assumed that they are related, at least three possibilities occur to us which might explain the negative *in vivo* results. (i) It may be necessary for each small molecular form of F. VIII to attach to a larger moiety in order to be active physiologically. Following transfusion in the dog, the coagulant-active component may be unable to combine with the F. VIII "carrier" protein (1, 9) due to abnormal binding sites on the latter. (ii) The "carrier protein" of the hemophilic dog might rapidly bind the coagulation-active peptide in a manner so sterically abnormal that it is rendered inacessible to the other reactants involved in normal coagulation (10). (iii) The small molecular entities may be so rapidly cleared from the circulation that an effect cannot be readily detected.

Some of these suggestions have been drawn from the experiments of Cooper *et al.* (11) who have demonstrated that when normal canine F. VIII is dissociated with 0.25 *M* $CaCl_2$ the active coagulant fraction does not recombine with the "carrier protein" of hemophilic dog plasma. This is, of course, the reverse of the results found with human fractions in which full recombination has been found to occur between dissociated normal F. VIII and the "carrier protein" purified from hemophilic plasma (11).

Our experiments with polyamino acids leave many questions unanswered. These include: (a) Why should PGA and PAA have *any* procoagulant activity? (b) Why should the procoagulant activities of PGA and PAA manifest themselves primarily as F. VIII, secondarily as F. IX, and not at all as F.'s V, VII, X, or XII? (c) Why is prior activation with kaolin an absolute requirement for assaying the activities of PGA and PAA? (d) Why is PGA active against the F. VIII defective plasma of man and not the F. VIII-defective plasma of dogs? (e) What is the significance of "potentiation."

Perhaps our experiments with the polypeptides are suggesting that F. VIII activity is a less specific phenomenon than is ordinarily thought, i.e., is less specific than the enzymatic activities of F. II, VII, IX, and X. If the "F. VIII effect" proves to be more complex and not as limited in specificity as that of the serine proteases, our experiments suggest that it may be possible someday to prepare artificial F. VIII.

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vations on Hexachlorophene-Induced Paralysis in the Cat and Its Antagonism by Hypertonic Urea (39352)

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toxic effects of hexachlorophene
on the central nervous system in-
paralysis of limbs with tonic extensor
and edema and spongy degeneration
te matter in the brain and spinal cord
These effects are reversible upon ces-
of HCP administration (2), making it
ly that permanent damage to cerebro-
neurons is sustained in surviving ani-
Instead, the signs suggest a "pressure
' (5) with the possibility of fatal out-
n cases that are severe, sustained, and
ved. This led us to investigate
er toxic amounts of HCP produce ele-
s of cerebrospinal fluid pressure
') and if so, whether this effect could
agonized or reversed. The wide range
rological observations accessible to
"clinical" test in the cat was the basis
oice of this species over the rat for
reported here.

erials and methods. Eighteen male
el cats, 1 to 2 years old, were immu-
for distemper and pneumonitis and
d to become accustomed to our labo-
for 4 to 5 weeks before use. They
removed from their cages twice each
and allowed free movement in the
l room while under observation to ac-
n them to the handling necessary for
and examination. They were system-
y observed before and after treatment
ling to a checklist that included the
a from Norton and DeBeer (6), in-
g recognition of personnel, grooming,
g, drinking, and exploratory behav-
d many of the neurological tests out-
y McGrath (7), such as muscle tone,
, righting, placing, and extensor and
reflexes.

P, at a dose of 20 mg/kg, was weighed
capsule for each animal and adminis-
laily by the oral route. Blood samples were taken at regular intervals and HCP concentrations in whole blood were measured by the gas-liquid chromatography method of Ulsamer (8).

Cats in complete paralysis were placed under pentobarbital anesthesia and cisternal CSFP was measured by a modification of the method of Hayes and Corey (9), using a Statam P-23 pressure transducer and a Grass Model 7 polygraph. A 3-way valve attached to the pressure transducer was used to calibrate the polygraph to a full scale deflection with a 100-mm column of 0.9% saline. Pressures were taken via a 22-gauge needle inserted into the cisterna magna. Paralyzed cats were given urea in invert sugar solution, acetazolamide, or prednisolone by slow iv infusion to investigate the possibility that toxic effects of HCP could be reversed. Upon termination of the experiments brain and spinal cord were collected for neuropathology.

Results. The number of daily doses required before the cats showed the first signs of toxicity (stiffness of the hind limbs and swinging movements of the pelvic girdle) varied from 2 to 12, and the number of additional doses required for the subsequent progression to severe toxicity varied from 1 to 4 (Table I). This wide variation seemed to be related to both differing individual animal sensitivities to HCP and to the wide variation in degree of absorption of HCP after oral administration. Blood concentrations determined at various times after each dose showed that HCP was both rapidly absorbed and rapidly cleared from the blood; values reached peaks ranging from approximately 3-17 μg/ml at or before 12 hr after each dose and then decreased to relatively low levels ranging from 0.1-0.9 μg/ml 24 hr after each dose (Table II). There was great variability in HCP concen-

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eserved.

TABLE I. A COMPARISON OF PATHOLOGY FINDINGS WITH THE NUMBER OF DOSES OF HEXACHLOROPHENE CAUSING ONSET OF TOXICITY AND CEREBROSPINAL FLUID PRESSURE IN THE CAT.[a,b]

	Number of doses			Pathology findings				Cerebrospinal fluid pressure (mm saline)	
Cat number	First toxicity	Severe toxicity	Total	Spinal cord	Brainstem	Cerebellum	Cerebral hemisphere	Before HCP	After HCP
27715	4	8	8	2	4	4	4	–	240.0
3285	4	7	7	4	4	4	4	–	135.0
CP-5[c]	–	–	–	n	n	n	n	–	50.0
CP-6[c]	–	–	–	n	n	n	n	–	82.5
28826	12	15	15	4	4	4	4	–	167.5
3249	–	–	8	1	2	2	2	–	132.5
3289	2	3	3	2	2	2	2	–	182.5
26400	9	13	13	2	4	4	4	–	185.0
3281	6	9	9	2	4	4	4	22.5	262.0
3291	6	7	8	1	4	4	4	27.5	115.0
5167	6	7	8	1	4	4	4	14.0	85.0
3292	5	6	7	n	4	4	4	22.5	120.0
3280	4	5	6	4	4	4	4	27.5	275.0
28606	4	5	6	4	4	4	4	17.5	185.0

[a] Pathology scores: 1 = slight focal vacuolization of white matter; 2 = slight focal or diffuse; 3 = moderate vacuolization with some displacement of architecture; 4 = moderate to severe; n = normal.

[b] The daily hexachlorophene dose was 20 mg/kg.

[c] CSFP was taken 1 month after the last HCP dose.

TABLE II. HEXACHLOROPHENE CONCENTRATIONS[a] IN WHOLE BLOOD OF CAT AT SEVERAL INTERVALS AFTER A SINGLE ORAL DOSE OF 20 mg/kg.

	Interval after dosing (hr)				
Experiment number	2.5	6.0	12.0	24.0	Number of cats
C-3	7.98 ± 1.29	7.63 ± 1.69	6.47 ± 1.76	0.89 ± 0.20	6
C-4	–	3.08 ± 0.59	3.93 ± 0.95	0.54 ± 0.15	5

[a] Expressed in μg/ml ± SEM.

trations in blood between cats and from day to day in the same animal. One major cause of the variation may be the fact that cats often vomited; when this occurred within 3 to 6 hr after HCP dosing much of the substance was lost and concentrations in blood were low. Since vomiting did not appear within a few minutes, it probably resulted from an action on the vomiting center and therefore may have been the earliest sign of toxicity within the neuraxis.

The toxic effects of HCP in the cat were seen as a gradually progressing degeneration of first motor and then autonomic functions that began in the hindquarters and proceeded craniad. The first signs were stiffness of the hind limbs that resulted in rigid gait and an elevated and widely swinging movement of the pelvic girdle. These signs later extended to include incoordination of all limbs, ataxia and weakness of the limbs, and occasionally patellar hyperreflexia and impairment of righting reflex. Sustained urinary retention also occurred and was often accompanied by hematuria, resulting in a greatly distended bladder that could be relieved by manual expression of urine. Signs suggesting pain, discomfort, and possibly disorientation appeared at this stage of toxicity and were accompanied by what might be called distressed crying; food consumption dropped to low levels with progressive and severe weight loss and signs of dehydration. As intoxication worsened it progressed to complete prostration; signs during this stage included loss of righting reflex, spastic and/or flaccid paralysis of hindlimbs, inability to stand or walk, and depressed or absent forelimb placement reactions followed by spastic paralysis of the forelimbs. The forelimb paralysis was often accompanied by an exaggerated extensor reflex elicited by lifting the head and stretching the neck dorsally. Cranial nerve functions remained unaffected; i.e., ocular accommodation, pupillary and eyelid reflexes, pinnal and vibrissal responses to persons and touch, reactions to "recognition" of environmental nnel, etc.) changes, and abdominal ıtion were normal, and the animals drink water when a container was ıt into contact with the mouth. In only ıse was there suggestion of unilateral r) papilledema. Finally, the animals ted complete flaccid paralysis, loss of lexes, dilated pupils, and hypothermia ed by death within 24 hr.

mals which were completely para- but which had not reached the final of CNS depression generally survived :P administration was discontinued. cats recovered slowly with the signs of :y disappearing in a sequence opposite t in which they developed. Neuropa- ;y performed at various stages of in- tion showed spongy degeneration or s spongiosis" of white matter in brain ɔinal cord without neuronal involve- there was no dilation of cerebral ven- or damage to ependymal linings. In ıl instances the white matter of the was more severely affected than that spinal cord. Complete recovery after ıal dose of HCP required 4 to 6 weeks : I).

s in complete paralysis were placed pentobarbital anesthesia and cisternal was measured. CSFP in these animals gnificantly elevated to a mean of 174 ıline, whereas untreated controls had n pressure of 19 mm saline (Tables I II). The elevated pressure in HCP- d animals began to fall several minutes ı slow iv infusion of 30% urea (in 10% sugar solution) at a dose of 2 g/kg and was rapidly reduced, within 1 hr, by a mean of 256 mm saline to a mean negative pressure of −100.1 mm saline relative to atmospheric pressure, which is 0 mm in our system (Table III). This effect lasted 3 to 4 hr during which time the elevation of CSFP gradually returned. Acetazolamide (200 mg/kg) given concomitantly with or after urea administration did not affect CSFP. In addition, neither acetazolamide nor prednisolone (2.5 mg/kg, iv) administered alone had any effect on the elevated CSFP in HCP-intoxicated animals.

LE III. EFFECT OF HEXACHLOROPHENE AND ON CEREBROSPINAL FLUID PRESSURE (CSFP) OF CATS.[a]

ment up	Mean CSFP ± SEM	Number of cats	P
	19.4 ± 2.3	8	
:ated	173.8 ± 17.5	12	<0.001[b]
:ated, urea on	−100.1 ± 27.7	6	<0.001[c]
e urea on	256.4 ± 10.9	6	

ɔressed as mm saline.

npared to control cats.

npared to HCP-treated cats.

In a series of separate experiments in which unanesthetized animals were visually observed, five cats paralyzed by HCP were able to stand and walk within 50 min after having been given urea (same dose as above) by slow iv infusion. This very striking effect lasted 3 to 4 hr before paralysis returned.

Discussion. The toxicity of HCP has been shown to be associated with spongy degeneration of the white matter of the brain and spinal cord in rats (2, 3). This degeneration is very similar to the "status spongiosis" seen in other disorders and thought to be due to diffuse brain edema (5, 10, 11). The data of the present report show that considerable elevations in cerebrospinal fluid (and therefore intracranial) pressure occurs with progressive paralysis in the HCP-poisoned cat, and that relief of the elevated pressure results in reversal of paralysis. We have shown that urea, an osmotic diuretic, lowers this elevated CSFP (12, 13), probably by removing fluid from the brain mass (14). Examination of brain sections from HCP-poisoned cats in the present study did not show dilation of the cerebral ventricles, ruling out the possibility of intracranial hypertension of obstructive origin. Thus it would appear that increased pressure does not arise from increased fluid in the ventricles, but rather in the surrounding tissue. In fact, the whole-brain sections in our study showed extensive and severe damage to white matter and myelin sheaths only, in agreement with the findings of others (2, 10, 15) who have observed demyelination and splitting, with no injury to the ependymal lining of the ventricles or the arachnoid villi. The marked elevation in CSFP produced by HCP and its relief by an osmotic agent is consistent with the reported recovery of rats from paralysis, upon cessation of HCP administration, so long as HCP had not damaged critical life-sustaining centers of the CNS. It is also consistent with the opinion that irreversible damage to CNS neurons need not occur to account for fatal paralysis; no neuropathology sections of brain and cord of cats dying from HCP in our studies showed any neuronal damage.

Finally, the inability of acetazolamide to alter significantly the elevated CSFP in HCP-poisoned cats appears to rule out a lesion resulting in overproduction of cerebrospinal fluid. Likewise prednisolone, commonly used to reduce intracranial pressure following trauma, did not affect (up to 6 hr) the elevated CSFP in HCP-poisoned cats. This was not entirely unexpected since the latency or onset of effect of this compound has been reported to be much longer than that of urea (16) and may be a significant limitation in its usefulness in treating HCP poisoning. Also, the failure of prednisolone to have an effect suggests that the HCP lesion may not be inflammatory in origin.

The limits of usefulness and the safety of urea and other osmotically acting drugs in treatment of HCP intoxication of life-threatening severity need to be established by additional study, since the results of the present study suggest that elimination of the administered urea under conditions of our tests results in reestablishment of the intracranial hypertension. It is possible that a constant iv infusion of urea might significantly prolong its actions. Edema of other organs may also be affected by urea; however, barring the occurrence of serious cardiac arrhythmia of sudden onset, this treatment may be capable of counteracting many supralethal doses of HCP. This might have important clinical implications for the management of cases of poisoning with this substance.

Summary. Cats given HCP (20 mg/kg) orally each day developed hindlimb paralysis and greatly elevated CSFP (174 mm saline; 19 mm in controls) in 3 to 5 days. "Status spongiosis" was seen in white matter only in sections of brain and cord. There was no dilation of cerebral ventricles, or damage to their ependymal linings or to the arachnoid villi. The neurological picture excluded any but a terminal effect upon cranial nerve function. There appeared to be no damage to neurons, and recovery of survivors was complete within 6 weeks after cessation of HCP administration. Elevated CSFP in paralyzed anesthetized cats was quickly lowered by an average of 256 mm by slow iv administration of 30% urea (2 g/kg in 10% invert sugar). Unanesthetized cats similarly paralyzed were able to stand and walk for up to 4 hr after this treatment. Neither acetazolamide nor prednisolone alone had any effect, nor did coadministration with urea enhance the effect of urea. The HCP lesion does not appear to be inflammatory in origin, nor does it seem to involve ventricular obstruction or overproduction of cerebrospinal fluid. The reappearance of paralysis about 4 hr after osmotic diuresis, which corresponds with the elimination of urea, suggests that prolonged iv infusion with urea or a similar osmotically active substance may have significant clinical value in the management of HCP poisoning.

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Ammonium Sulfate Activation of Phosphodiesterase in Homogenates of Larvae of the European Corn Borer (39353)

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We first noted activation by high concentrations of ammonium sulfate of phosphodiesterase(s) found in crude and partially purified extracts of larvae of the European corn borer, *Ostrinia nubilalis* (Hübner), after the ammonium sulfate precipitation step when we attempted to purify cyclic adenosine monophosphatase (c-AMP-ase), phosphomonoesterase, and phosphodiesterase by the method of Robison (1). Although neither the ammonium nor the sulfate ions are of major importance in the activation of many enzymes, several workers have reported that ammonium ion enhances the activity of β-D-galactosidase (2) and the deamination of L-serine by the β-subunit of tryptophane synthetase (3). Also, the ammonium ion activates lactose hydrolysis (4), AMP-aminohydrolase (5), and D-β-lysine mutase (6). Moreover, in the absence of potassium ions, β-lysine mutase is activated by ammonium ions; if potassium ions are present, the activity is inhibited (6). Ammonium ions can substitute for one subunit of tryptophane synthetase from *Escherichia coli* (7), and nucleoplasmin, divalent manganese ions, and ammonium sulfate together activate RNA polymerases derived from eukaryotic nuclei (8–13). Kastenschmidt *et al.* (14) demonstrated activation of phosphorylase b from rabbit skeletal muscle by AMP. This activation was enhanced by sulfate and fluoride ions when the reaction mixture was made up in glycylglycine. They suggested that sulfate ion enhanced the interaction of AMP with phosphorylase. Appleman found that inactive phosphorylase from liver is not activated by AMP unless a salt solution such as sodium sulfate is added (15). Sulfate ions also activate sulfatases (16).

Since one goal of our work is to determine differences in metabolism between insect tissues and other invertebrate and mammal tissues, in the present study we determined phosphodiesterase levels in at least one other arthropod (the crayfish), in two ruminants, and in a rat. We believe that ours is the first report describing activation by ammonium sulfate of phosphodiesterase(s) prepared from insect tissue.

Materials and methods. The European corn borer larvae used in the study were supplied by the European Corn Borer Laboratory, ARS, USDA, Ankeney, Iowa. The insects were maintained in our laboratory as described by Odesser *et al.* (17). The adult bovine brain and liver were supplied by Richard W. Miller of our laboratory, the yearling ovine liver, kidney, and brain tissue by the Ruminant Nutrition Laboratory, the rat livers from the Carbohydrate Laboratory and 3-in. Louisiana crayfish, *Cambarus* sp., by the Pesticide Degradation Laboratory (all Beltsville Agricultural Research Center, USDA, Beltsville, Md.)

The phosphodiesterase and phosphomonoesterase were assayed as described by Odesser *et al.* (17) except that phenylthiourea, which prevents browning of insect extracts, was omitted from the extraction fluid. When ammonium sulfate was tested, it was added directly to the incubation tubes before the 1.4 ml of bis-Tris–$MgCl_2$ was added. All tubes were centrifuged at 25,000 rpm for 30 min before colorimetric determination. All reagents were C.P. grade or better.

Results and discussion. Figure 1 shows the effect of the ammonium sulfate concentration on hydrolysis of bis(*p*-nitrophenyl) phosphate. Maximum phosphodiesterase activity was obtained when the reaction mixture was 30% saturated with respect to ammonium sulfate as illustrated in Fig. 1. Precipitation of diesterase was a major factor in the observed decrease of enzyme activity above 30% saturation since ammonium sul-

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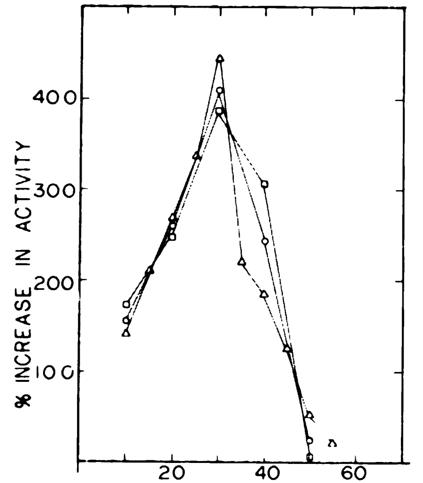

FIG. 1. Activation of crude phosphodiesterase preparation by ammonium sulfate. Reaction vessel contains, in addition to ammonium sulfate as indicated, 0.6 ml of enzyme preparation containing 15-18 mg of tissue (wet wt) in sucrose (0.25 *M*); phenylthiourea (0.1 saturation); bis(*p*-nitrophenyl) phosphate (6 μmole in 0.6 ml of Tris [(hydroxymethyl)amino]-methane buffer (Tris); 0.2 *M*, pH 8.4); 0.6 ml of Tris buffer (0.2 *M*, pH 8.4); 0.2 ml of $MgCl_2$ (1 *M*). □, Trial 1; △, Trial 2; ○, average of two trials.

fate is used to precipitate phosphodiesterase during purification of the enzyme (1).

The reason for the 400-fold activation of phosphodiesterase could possibly be one or more of the following:

1. Precipitation of magnesium ammonium phosphate could drive the reaction as follows: [a] bis(*p*-nitrophenyl) phosphate→*p*-nitriphenyl phosphate + *p*-nitrophenol; [b] *p*-nitrophenyl phosphate → P_i + *p*-nitrophenol; [c] $P_i + Mg^{+2} + NH_4^+ \rightarrow$ magnesium ammonium phosphate.
2. Precipitation of a high molecular weight inhibitor.
3. Action as a cation and anion activator of the enzyme.
4. Alteration of the tertiary or quaternary structure of the enzyme so that a more active form was obtained.
5. Action resulting from some combination of the preceding possibilities.

The results of a test of the first possibility were negative. Addition of magnesium chloride made no difference in the activation by ammonium sulfate; with magnesium present activity was 47.8 μmoles nitrophenol released, and with magnesium absent activity was 49.8 μmoles nitrophenol released in a whole homogenate; in a 105,000 *g* supernatant values with and without magnesium were, respectively, 9.0 and 10.1 μmoles liberated.

A decrease in magnesium concentration should have resulted in a decrease in diesterase activity. Also, the addition of ammonium sulfate should have resulted in increased monoesterase activity, since it is this reaction which generates the inorganic phosphate ion (see Eq. [b]). In two trials phosphomonoesterase activity was inhibited by ammonium sulfate (30% satd.); from 13.1 to 8.6 and 13.9 to 11.0 μmoles nitrophenol liberated/g wet wt corresponding to 35 and 21% inhibition, respectively.

Another test was made to determine whether a permanent change occurred in the configuration of the enzyme or whether an inhibitor was precipitated. Thus we added ammonium sulfate to the larval extract at 30% saturation, centrifuged half the mixture at 5000 *g* for 20 min at room temperature and held the other half at room temperature for the same length of time. Both samples were then dialyzed at 40° overnight against the homogenizing fluid (sucrose, 0.25 *M*) which in this case was 0.1 saturated with phenylthiourea. After dialysis the diesterase activity of each sample was determined. The activities of the two samples were not different from each other nor were they different from the activity of untreated homogenates. We therefore concluded that an inhibitor had not been precipitated and that no permanent change had occurred in the configuration of the enzyme. Figure 2 shows that another ammonium salt, ammonium chloride and another sulfate, sodium sulfate, did activate the enzyme although not to the same extent as ammonium sulfate. These data suggest that ammonium and sulfate together are more effective than either ion alone in activating the enzyme.

The possibility that activation might have been the result of increased salt concentration was tested. The ionic strength of 10% sodium sulfate is 2.10; the ionic strength of 30% saturated ammonium sulfate is 4.05.

The ionic strength of 30% ammonium chloride was 6. These concentrations had been chosen on a somewhat arbitrary basis; for example, proteins are more readily precipitated in sodium sulfate than in ammonium sulfate and this had been considered in designing the tests. A test to determine the effects of sodium chloride and ammonium sulfate on enzyme activity when ionic strengths were approximately equivalent is illustrated in Table III. Here the salt effects of NaCl appear to increase activity about 50%, similar to effects obtained with ammonium chloride, i.e., about 50%. The effect was distinctly less than the effect observed in ammonium sulfate in which activity was increased either 248 or 463%, depending upon the concentration.

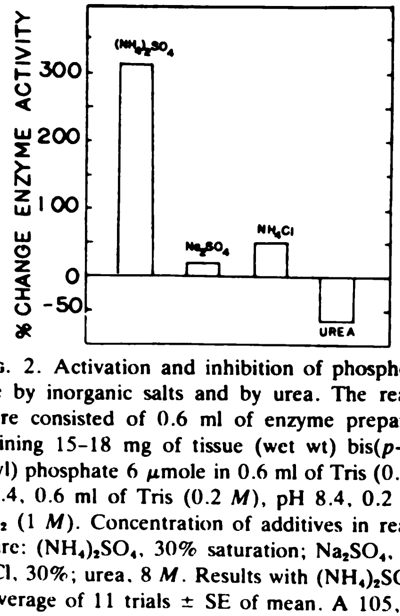

FIG. 2. Activation and inhibition of phosphodiesterase by inorganic salts and by urea. The reaction mixture consisted of 0.6 ml of enzyme preparation containing 15-18 mg of tissue (wet wt) bis(*p*-nitrophenyl) phosphate 6 μmole in 0.6 ml of Tris (0.2 *M*), pH 8.4, 0.6 ml of Tris (0.2 *M*), pH 8.4, 0.2 ml of $MgCl_2$ (1 *M*). Concentration of additives in reaction mixture: $(NH_4)_2SO_4$, 30% saturation; Na_2SO_4, 10%; NH_4Cl, 30%; urea, 8 *M*. Results with $(NH_4)_2SO_4$ are the average of 11 trials ± SE of mean. A 105,000 *g* supernatant was used in the test with ammonium chloride.

We anticipated that 8 *M* urea might activate the enzyme since it disrupts tertiary structures. If high concentrations of ammonium sulfate were required, 30% saturation could produce alteration in the tertiary or quaternary structure. This was not the case as shown in Fig. 2. The 8 *M* urea solution inhibited activity 67%, probably by disrupting tertiary structure. Since Winstead and Wold (18) observed that 0.5 *M* ammonium sulfate in 20% aqueous dioxane inhibited enolase by causing dissociation into subunits, the activation we observed could possibly have depended upon dissociation of our enzyme into subunits that were more active than a larger enzyme molecule.

Our most reasonable explanation of the ammonium sulfate effect is that there was activation by both ammonium and sulfate ions. However, further work with purified enzyme will be required to determine whether this interpretation is correct.

Subsequently the addition of ammonium sulfate to 30% saturation was found to decrease the pH of our incubation mixture to 7.6. Phosphodiesterase activity at pH 7.6 and 8.4 was not different in the crude preparations for in six samples run at each pH value the average activity was 11.5 ± 5.3 (pH 8.4) and 11.5 ± 4.9 (pH 7.6).

In our previous paper (17) we demonstrated that preparations from insect sources exhibited phosphodiesterase activities quite different from the phosphodiesterase found in snake venoms. The results shown in Tables I and II indicate that this is true in other species as well, since ammonium sulfate inhibited or did not affect rather than activated the phosphodiesterase of all samples tested (Table I). Also, Table II shows that the inhibitors, caffeine and thiophylline, inhibited some mammalian phosphodiester-

TABLE I. INHIBITION OR ACTIVATION OF PHOSPHODIESTERASE ACTIVITY OF HOMOGENATES OF SELECTED VERTEBRATE AND INVERTEBRATE TISSUE BY $(NH_4)_2SO_4$ AT 30% SATURATION.

	p-Nitrophenol released		
Organ	μmole/g wet wt + $(NH_4)_2SO_4$	μmole/g wet wt − $(NH_4)_2SO_4$	Change (%)
Rat liver (1)[a]	7.8	6.8	−13
Bovine liver (1)	11.4	5.3	−54
Bovine kidney (1)	8.5	7.8	−8
Crayfish digestive gland (2)	37.6	32.6	−13
Whole European corn borer larvae (2)	8.3	46.8	+463

[a] Number of determinations in parenthesis.

BLE II. THE EFFECT OF CAFFEINE (0.008 *M*)[a] AND THEOPHYLLINE (0.008 *M*)[a] ON PHOSPHODIESTERASE ACTIVITY OF SELECTED VERTEBRATE AND INVERTEBRATE TISSUES.

Organ	Untreated homogenate *p*-Nitrophenol released (μmole/g wet wt.)	Percentage difference in level of enzyme activity after addition to reaction mixture of: Caffeine[c]	 Theophylline[c]
Rat liver (1)[b]	13.4	−4.5	−15.7
Bovine liver (1)	33.0	−7.6	−23.1
Bovine brain (1)	1.6	−25.0	−39.3
Ovine liver (2)	16.6	−15.8	−23.6
Ovine kidney (2)	27.6	−11.3	−46.2
Ovine brain (2)	1.8	−16.8	−22.3
Crayfish digestive gland (2)	21.6	−4.0	+11.8
Crayfish green gland (1)	28.6	−6.3	−17.9

inal concentration in reaction mixture.

umber in parentheses indicates number of trials.

desser *et al.* (14) found 51 and 60% inhibition by caffeine (0.118 *M*) and theophylline (0.008 *M*), tively, of phosphodiesterase in homogenates prepared from fifth instar European corn borer larvae.

TABLE III. EFFECT EQUIVALENT OSMOLAR ONCENTRATIONS OF NaCl AND $(NH_4)_2SO_4$ ON SPHODIESTERASE ACTIVITY[a] IN HOMOGENATES OF EUROPEAN CORN BORER LARVAE.

t added	Osmoles of salt added	*p*-Nitrophenol released/g wet wt (μmole)	Increase in activity (%)
ne	0	8.3	–
Cl	1.95	12.7	53
$H_4)_2SO_4$	1.95	28.9	248
Cl	3.90	13.0	57
$H_4)_2SO_4$	3.90	46.8	463

The reaction mixture contained 0.6 ml of enzyme
aration containing 15–18 mg of tissue (wet wt); bis
trophenol) phosphate, 6 μmole in 0.6 ml of Tris
M), pH 8.4; 0.6 ml of Tris (0.2 *M*), pH 8.4; 0.2 ml
gCl_2 (1 *M*). Either NaCl or $(NH_4)_2SO_4$ was added
at final solution contained 1.95 or 3.90 osm of the

; slightly, but the levels of inhibition
e far lower than the 51% inhibition (caf-
e) and 60% (theophylline) reported for
opean corn borer diesterase (17). Also,
ther arthropod, the crayfish, had dies-
ses that behaved differently from our
yme, since the phosphodiesterase of the
stive gland (hepatopancreas) was acti-
d by caffeine in the concentrations used.
), the phosphodiesterase of the green
d (antennal gland, renal-type function)
hibited 6.3% in the presence of caffeine
17.9% in the presence of theophylline
ble II). This suggested that the phospho-
terases of the European corn borer were qualitatively different from those in other species examined in this study.

Activation of the corn borer enzyme by ammonium sulfate may provide an explanation for an earlier, previously unreported observation made in our laboratory. We found that when 0.03 *M* ammonium chloride was injected into European corn borer larvae, it caused death, while topical treatment of pupae of another species of Lepidoptera, the tobacco budworm, *Heliothis virescens* (F.), with ammonium chloride also resulted in high mortality. The toxicity could be a result of activation of the phosphodiesterase(s) that attack diphosphates *in vivo*. Further studies may suggest ways in which activation of phosphodiesterases could be used to manipulate harmful pest populations.

Summary. European corn borer phosphodiesterase is highly activated by $(NH_4)_2SO_4$ and moderately activated by NH_4Cl (pH 7.6, 33°). Vertebrate and crayfish diesterases, on the other hand, are inhibited by $(NH_4)_2SO_4$. It is likely that $(NH_4)_2SO_4$ causes some configurational change in the European corn borer phosphodiesterase molecule which results in the exposure of more active sites and hence greater enzyme activity. In *in vitro* tests caffeine (0.008 *M*) and theophylline (0.008 *M*) inhibit phosphodiesterase more effectively in European corn borer larvae than in crayfish, ovine, bovine, or rat tissue.

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Effect of Adenosine 3′,5′-Monophosphate on Serine Metabolism in Chick Tissues (39354)

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is research suggest control mecha-
glycine and serine metabolic en-
ffer for rats and chicks (1). An
on for this species difference in cell
n of serine enzymes is the large
f glycine or serine needed for uric
uction in chicks. Seventy-five per-
ein diets fed to chicks produced
ls of serum uric acid and also in-
ctivities of serine biosynthetic en-
-phosphoglycerate dehydrogenase,
sphoserine phosphatase, in chick
ereas activity for the catabolic en-
rine dehydratase, remained the
npared to control chicks (1). This
n protein diet fed to rats has been
increase serine dehydratase activ-
liver and simultaneously decrease
oglycerate dehydrogenase and
erine phosphatase activities (2, 3).
novo synthesis of serine dehydra-
occurs after intraperitoneal injec-
cyclic AMP in rats (4). The in-
enzyme activity is completely
f Actinomycin D is administered
rior to the injection of the cyclic
e. High protein carbohydrate free
e been shown to increase cyclic
els in perfused rat liver (5), hence
g the two experimental parame-

biosynthetic enzyme activities are increased in hepatic tissue of chicks fed crystalline amino acid diets supplemented with 2% glycine or serine compared to enzyme activities from chicks fed no glycine or serine (6). Oral intubation studies with rats suggest glycine or serine play an important role increasing the transport of cyclic AMP in liver tissue (7).

The present investigation was initiated to determine the *de novo* synthesis of serine metabolic enzymes in chicks fed crystalline amino acid diets with supplemental glycine and in chicks given high doses of cyclic 3′,5′-AMP by intraperitoneal injections.

Materials and methods. One-hundred-day-old broiler chicks were distributed at random into five groups of 20 chicks each. The chicks were placed in electrically heated wire batteries and fed *ad libitum* an amino acid diet deficient in glycine and L-serine (Table I) for 3 days. Two groups of chicks were then fed the basal diet with 2% supplemented glycine, and the remaining three groups were fed the basal diet deficient in glycine and L-serine. Chicks in group 1 were fed the diet with 2% supplemented glycine and were intraperitoneally injected daily with 1 ml of 0.9% NaCl. The chicks in group 2 were fed the 2% glycine supplemented diet and were injected daily with 0.5 ml of Actinomycin D (10 μg/ml). The chicks of groups 3, 4, and 5 were fed the basal diet and were intraperitoneally injected with 1 ml of 0.9% NaCl, 0.5 ml of cyclic 3′,5′-AMP (5 mg/ml), and 0.5 ml of Actinomycin D (10 μg/ml) + 0.5 ml of cyclic 3′,5′-AMP (5 mg/ml), respectively, for a 5-day period. The chicks in group 5 fed the basal diet were injected intraperitoneally with Actinomycin D, 30 min prior to the injection of cyclic 3′,5′-AMP. The birds were sacrificed on Day 8 and livers were removed for enzyme determination.

Enzyme preparation and assay. Chicks

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per is a part of the dissertation which was
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Maryland 20852.

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TABLE I. COMPOSITION OF AMINO ACID DIETS.

Basal diet	
Ingredient	g/kg
L-Valine	10.00
L-Leucine	14.00
L-Isoleucine	8.00
L-Threonine	7.00
L-Glutamic acid	100.00
L-Tryptophan	2.25
DL-Methionine	7.00
L-Cystine	3.50
L-Phenylalanine	7.00
L-Tyrosine	7.00
L-Lysine · HCl	14.00
L-Arginine · HCl	14.00
L-Histidine · HCl	4.50
L-Proline	10.00
Corn oil	90.00
Antiacid[a]	5.00
Salts mixture[b]	60.00
Cellulose[c]	30.00
$NaHCO_3$	10.00
Choline chloride	2.00
Vitamin mix[d]	5.00
Vitamin E supplement (10 mg/g)	1.00
Glucose monohydrate[e]	588.75

[a] Antiacid: 3 parts $Mg_2Si_3O_8$ and 1 part $Al(OH)_3$.

[b] The salt mixture supplied the following reagent minerals (g/kg diet): $NaMoO_4 \cdot 2H_2O$, 0.01; $CuSO_4$, 0.02; KI, 0.05; $ZnCO_3$, 0.20; $FeC_6H_5O_7$, 0.40; $MnSO_4 \cdot 2H_2O$, 0.42; KCl, 3.0; $MgSO_4 \cdot 7H_2O$, 5.0; NaCl, 6.00; Na_2HPO_4, 7.20; KH_2PO_4, 12.0; $Ca_3(PO_4)$, 13.95; and $CaCO_3$, 15.00.

[c] Solka Floc, Brown Company, Berlin, New Hampshire.

[d] The vitamin mixture supplied the following vitamins (mg/kg diet): thiamine · HCl, 100.0; niacin, 100.0; riboflavin, 16.0; D-calcium pantothenate, 20.0; cyanocobalamin (mg/g), 20.0; pyridoxine · HCl, 6.0; biotin, 0.6; folic acid, 4.0; inositol, 100.0; menadione sodium bisulfite, 5.0; retinyl palmitate, 10,000 IU; and cholecalciferol, 1200 ICU.

[e] Cerelose, Corn Products Company, Argo, Illinois.

were sacrificed by cervical dislocation and livers were removed and weighed. Liver tissues were prepared and assayed for D-3-phosphoglycerate dehydrogenase (EC 1.1.1.s), phosphoserine phosphatase (E.C. 3.1.3.3) and L-serine dehydratase as described by Fallon *et al.* (8). Glycerate dehydrogenase (EC 1.1.1.29) was determined by the method of Willis and Sallach (9). The enzyme assay procedures were each adjusted for chick tissue to allow for linear initial velocity studies.

Protein determinations were made according to the method of Lowry *et al.* (10) using crystalline bovine serum albumin as a standard.

Results and discussion. The specific activity of D-3-phosphoglycerate dehydrogenase and phosphoserine phosphatase was increased in liver tissue from chicks injected daily with 3',5'-AMP above that of those fed the same basal diet and injected with physiological saline (Table II). The specific activity of these two enzymes was increased in liver tissues from chicks fed the basal diet supplemented with 2% glycine, and injected with 0.9% NaCl above that of liver tissue from chicks fed the basal diet and injected with saline. Actinomycin D completely inhibited the increase in enzymatic activity of D-3-phosphoglycerate dehydrogenase in liver tissue from chicks injected with cyclic 3',5'-AMP or fed the basal diet supplemented with 2% glycine. These data suggest a *de novo* synthesis of D-3-phosphoglycerate dehydrogenase and phosphoserine phosphatase in liver tissue of chicks injected with cyclic 3',5'%-AMP or fed a basal amino acid diet supplemented with 2% glycine.

L-Serine dehydratase and glycerate dehydrogenase content of liver tissue were not significantly affected by the injection of cyclic 3',5'-AMP or the addition of 2% glycine to the amino acid basal diet (Table II).

The regulation of serine biosynthetic enzymes in chick liver tissue by cyclic 3'5'-AMP is a reversal of conditions observed in mammals (3).

It was previously mentioned that the metabolic enzyme, L-serine dehydratase is induced by the intraperitoneal injections of cyclic 3',5'-AMP in rats. It is possible that cyclic 3',5'-AMP is the regulator of serine catabolism in rats and serine synthesis in chicks. The possible difference in enzyme regulation in liver tissue by cyclic 3',5'-AMP may be due to the essential nature of glycine or L-serine for the chick. The *de novo* synthesis of serine biosynthetic enzymes in liver tissue from chicks fed a basal amino acid diet supplemented with 2% glycine may be due to the availability of cyclic 3',5'-AMP to the hepatic tissue.

Summary. The effect of cyclic 3',5'-AMP and supplemental dietary glycine upon *de novo* synthesis of serine metabolic enzymes in chick livers were examined. Chicks fed crystalline amino acid diets containing 2% glycine had approximately twofold the activ-

ABLE II. The Effect of Cyclic 3',5'-AMP and Actinomycin D on D-3-Phosphoglycerate Dehydrogenase, Phosphoserine Phosphatase, Glycerate Dehydrogenase, and L-Serine ehydratase Activity in Liver of Chicks Fed Various Proportions of Glycine for 5 Days.

		Specific activity[a]			
		D-3-PGD[b]	PP[c]	PGD[d]	S-D[e]
:t	Daily treatment	mμ Moles NAD/mg protein/hr × 10^{-3}	mμ Moles Pi/ mg protein/ hour	mμ Moles NADH/mg protein/hour	mμ Moles NAD/mg protein/hour
	1 ml 0.9% NaCl	13.8 ± 0.3c	67 ± 7b	304 ± 23a	70 ± 4a
+ 2% ie	1 ml 0.9% NaCl	25.4 ± 0.4a	130 ± 9a	326 ± 21a	69 ± 5a
+ 2% ie	5 μg Actinomycin D	14.3 ± 0.3c	75 ± 5b	312 ± 17a	69 ± 6a
	2.5 mg of cyclic AMP	23.5 ± 0.5b	123 ± 10a	309 ± 18a	71 ± 6a
	5 μg Actinomycin D + 2.5 mg cyclic AMP	13.3 ± 0.4c	63 ± 6b	291 ± 14a	68 ± 4a

ıns of three observations/group ± SEM. Each observation consisted of tissue samples from five chicks.
ith a common lettered subscript in a vertical row are not statistically different ($P < 0.05$).
Phodphoglycerate dehydrogenase (EC 1.1.1.s).
sphoserine phosphatase (EC 3.1.3.3).
lycerate dehydrogenase (EC 1.1.1.29).
ne dehydratase (EC 4.2.1.13).

iver for 3-phosphoglycerate dehydro-
and phosphoserine phosphatase
red to liver tissue from chicks fed
ıcking in dietary glycine. Chicks sub-
to daily intraperitoneal injections of
3',5'-AMP and fed diets containing
tary glycine contained biosynthetic
e activity similar to glycine-fed chicks
ting a correlation between glycine
clic AMP for serine enzyme induc-
he elevated enzyme activity in liver
ks fed dietary glycine or injected with
AMP was inhibited when chicks were
jected with Actinomycin D indicating
ɔ synthesis of 3-phosphoglycerate de-
enase and phosphoserine phospha-
)ietary glycine or cyclic AMP, how-
id not change serine dehydratase and
te dehydrogenase activities in chick

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 178-182 (1976)

Longitudinal Tension of Anterior Tibial Artery Segments during Constrictor Responses[1] (39355)

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Introduced by C. H. Baker

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We have previously shown (1, 2) that isolated segments of anterior tibial arteries of the dog underwent marked decreases in diameter when stimulated via their sympathetic innervation. The purpose of the present study was to determine if appreciable changes in longitudinal tension occurred in these vessels during vasoconstrictor responses. If longitudinal tension is increased during vasoconstrictor responses, appreciable changes in the architecture of the vascular bed could occur. Data on longitudinal tension changes occurring during vasoconstriction could also provide further insight into vascular smooth muscle mechanics.

Methods. Experiments were conducted on normally tethered and normally innervated anterior tibial artery segments of dogs. They were premedicated with morphine (3-5 mg/kg) anesthetized with sodium pentobarbital (20 mg/kg) and heparinized at 5 mg/kg. Arterial segments were isolated from the remainder of the limb vasculature by inserting nylon catheters upstream and downstream in the vessel and advancing the catheters until their tips were approximately 1 cm apart. This procedure produced a length of anterior tibial artery which was free of collateral channels except for vasa vasorum.

Segments were perfused with blood from the femoral artery of the contralateral leg with a constant output pulsatile pump. Outflow from the isolated segment was returned to the femoral vein of the contralateral limb. Resting intraluminal pressures were adjusted by a variable resistance on the outflow tubing. The perfusion circuit has been described in more detail previously (2).

Arterial segment inflow pressure was recorded through the side arm of a plastic T-tube with a P23G Statham pressure gauge. The T-tube was placed in the inflow circuit just upstream from the nylon catheter and was firmly positioned to prevent movement or stress from being exerted on the vessel segment. Outflow pressures were recorded through another plastic T-tube placed just distal to the outflow nylon catheter in the outflow circuit. The outflow T-tube was connected through a length of small wire to a Grass FT03 force transducer to record changes in axial tension. Springs were removed from the force transducer to provide maximum sensitivity. Care was taken to keep the blood vessel segment at its original length.

Vasoconstrictor responses were produced by stimulation of the superficial fibular nerve, by infusion of levarteranol at 1-2 μg/min into the inflow circuit, and by stimulation through platinum wire electrodes inserted into the blood stream at the inflow and outflow T-tubes. Succinylcholine chloride (2 mg/kg) prevented skeletal muscle contraction during superficial fibular nerve stimulation. Vasoconstrictor responses were recorded under constant inflow perfusion, under constant pressure perfusion, and at constant intraluminal pressure under constant pressure perfusion when the outflow circuit was occluded. Changes in longitudinal tension were also recorded when intraluminal pressures were passively changed by altering the outflow resistance under constant inflow perfusion.

Under constant inflow perfusion the entire output from the constant output pump was directed through the vascular segment. With inflow held constant, the inflow pressure and the pressure drop across the segment varied directly with vessel segment resistance. Under constant pressure perfusion a side arm containing a Starling resistor was opened to the inflow tubing at a site be-

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tween the pump and the vessel segment. As the resistance of the isolated vessel segment increased during vasoconstrictor responses, a greater portion of the pump output flowed through the parallel circuit containing the Starling resistor. Thus, small changes in vessel inflow pressure occurred during vasoconstrictor responses under constant perfusion. Complete occlusion of the outflow tubing under constant pressure perfusion directed all the output from the pump through the parallel circuit containing the Starling resistors with no flow or pressure change in the isolated vessel segment as it underwent constriction.

Results. Figure 1A shows inflow and outflow pressures and longitudinal tension changes of an isolated segment during superficial fibular nerve stimulation (15 Hz) under constant inflow perfusion. Sympathetic stimulation produced marked vasoconstriction as indicated by the increase in inflow pressure from a control level of 115 to 300 mm Hg at peak response. Outflow pressure, as would be expected under constant inflow perfusion, showed no significant change. The pressure gradient across the segment increased from 15 to 200 mm Hg as a result of increased vessel resistance. The longitudinal tension decreased by approximately 2 g.

Figure 1B shows the response of the same preparation to superficial fibular nerve stimulation under constant pressure perfusion at the same stimulation parameter as in Fig. 1A. Sympathetic stimulation produced a small increase in inflow pressure of 10 mm Hg. Outflow pressure decreased by 30 mm Hg, thus a small decrease in mean pressure occurred. A small decrease in longitudinal tension during the stimulation period was followed by an increase during the recovery period. Thus a relatively small change in longitudinal tension occurred during vasoconstriction when changes in intraluminal pressure were small.

Figure 2A illustrates the effect on longitudinal tension of passively increasing intraluminal pressure by partially occluding the outflow tubing under constant inflow perfusion. The increased intraluminal pressure produced a passive dilation of the vessel segment as indicated by the decreased difference between inflow and outflow pressures. Longitudinal tension decreased markedly with increased intraluminal pressure.

Figure 2B shows the response of the same segment to superficial fibular nerve stimulation under constant pressure perfusion, with the outflow tubing occluded to prevent any change in intraluminal pressure. Sympathetic stimulation then produced insignificant changes in longitudinal tension, although the diameter of the vessel was pre-

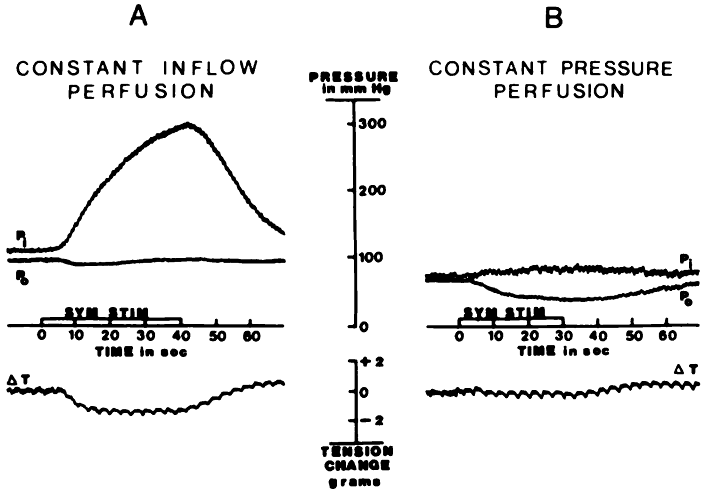

FIG. 1. Panel A shows response to superficial fibular nerve stimulation at 15 Hz, under constant inflow perfusion. Panel B shows response to same stimulus, under constant pressure perfusion. P_i and P_o represent inflow and outflow pressures, respectively. T represents changes longitudinal tension.

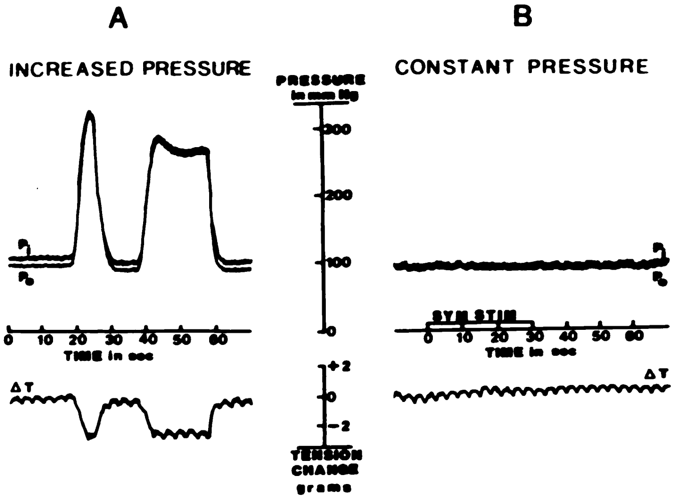

FIG. 2. Panel A shows response to partial occlusion of the outflow tubing under constant inflow perfusion. Tracings are arranged as in Fig. 1. Panel B shows response to superficial fibular nerve stimulation under constant intraluminal pressure. Constant intraluminal pressure was achieved by completely occluding outflow tubing under constant pressure perfusion.

sumably decreased as in the previous constrictor responses.

Five of the seven animals exhibited responses similar to those described for Figs. 1 and 2. The remaining two animals showed a greater tendency toward increased longitudinal tension during vasoconstrictor responses as shown by increases in longitudinal tension during constrictor responses under constant inflow and constant pressure perfusion. Changes in longitudinal tension of arterial segments from each animal to sympathetic stimuli, infusions of levarterenol, and increases in intraluminal pressure are shown in Table I. The mean of responses to each procedure for each animal are shown. Tension changes are expressed in grams for each procedure except for the increases in intraluminal pressure where directional changes were indicated. As will be described in the Discussion, these differences in response may depend on variations in orientation of smooth muscle elements in individual arteries. Similar changes in longitudinal tension as described for those obtained in response to superficial fibular nerve stimulation were obtained during infusions of levarteranol and during stimulation via electrodes in T-tubes upstream and downstream from the vessel.

Discussion. Relatively small changes in longitudinal tension occurred during vasoconstriction responses of arterial segments. Tension changes were in some cases positive and in other cases negative. Tension changes appeared to be the result primarily of changes in intraluminal pressure. The importance of intraluminal pressure changes was indicated by the relatively large decreases in longitudinal tension produced by passively increasing intraluminal pressure, and by the fact that during vasoconstrictor responses longitudinal tension changes varied with the magnitude of the intraluminal pressure change. During vasoconstrictor responses under constant inflow perfusion, with elevated intraluminal pressures, longitudinal tensions usually decreased. Under constant pressure perfusion and when intraluminal pressure was held constant, small changes in longitudinal tension occurred.

The results of the present study are substantiated by previous comparisons of circumferential and longitudinal stress relationships of blood vessels. Little information has, however, been available on longitudinal tension or length changes occurring during vasomotor responses. Most information applicable to this problem comes from comparisons of distensibility characteristics of relaxed and constricted blood vessels in *in vitro* preparations. Dobrin and Doyle (3, 4)

TABLE I. LONGITUDINAL TENSION CHANGES OF ANTERIOR TIBIAL ARTERY SEGMENTS.

	Sympathetic stimulation[a]			Levarterenol infusion[b]			Increased intraluminal pressure	
Dog number	Perfusion method[c]	Tension change in grams[d]	Number of times studied	Perfusion method	Tension change in grams	Number of times studied	Tension change in grams	Number of times studied
1	CIP	−2.3	3	CIP	− 2.5	3		
2	CIP	−1.4	2				−	3
	CPP	0	3					
3	CIP	+0.6	5	CIP	+ 0.9	1	−	1
	CPP	+0.3	2					
4	CIP	−1.4	1				−	1
	CPP	0	3					
5	CIP	−0.9	2				−	1
	CPP	−0.3	3					
6	CIP	+1.3	1	CIP	+ 1.8	1	−	1
		−0.7	1	CPP	+ 1.8	2		
	CPP	+0.4	1					
		−0.6	2					
7	CIP	−0.7	3	CIP	− 0.5	2	−	1
	CPP	−1.0	2	CPP	− 0.3	3		

[a] Produced by stimulation of the superficial fibular nerve or by electrodes inserted into blood stream as described in text.

[b] Levarterenol infused into inflow circuit at rate of 1-2 μg/min.

[c] CIP denotes constant inflow perfusion; CPP denotes constant pressure perfusion.

[d] (−) denotes decreased longitudinal tension; (+) denotes increased longitudinal tension; and (0) denotes negligible change in longitudinal tension. Values represent mean for that animal.

from studies on excised dog carotid arteries reported that activation of smooth muscle elements did not significantly alter the longitudinal elastic modulus. They reported that total longitudinal stress could be calculated as the sum of two components. One was the longitudinal stress due to pressure. The other was attributed to longitudinally applied traction. Vonderlage (5) from studies on strips cut at different angles from abdominal aorta of rabbits reported that distensibility characterisitics of strips cut parallel to the longitudinal axis were changed little after treatment with arterenol.

In our experiments both the circumference and length of vessels were increased by passive increases in intraluminal pressure. Appreciable increases in circumference were indicated by the decreased difference between inflow and outflow pressures under constant inflow as intraluminal pressure was passively elevated. Increased vessel length was indicated by the abrupt fall in longitudinal tension with increased intraluminal pressure. Previous studies also substantiate these findings. Dobrin and Rovick (6) and Bergel (7) showed that circumferential strains over physiological pressure ranges could produce increases of approximately 100% in vessel radius. Although the problem seems to be still unresolved as to whether or not arterial vessel walls are exactly isotropic (4, 7, 8) these studies indicate that increases in intraluminal pressures produce increases in length as well as in circumference (9).

Little information is available on morphological changes of vascular smooth muscle elements during contraction. If smooth muscle elements were arranged in helical coils around the circumference of the vessel, shortening should produce a tension vector parallel to the long axis of the vessel with a resultant increase in longitudinal tension. On the other hand, circumferentially oriented smooth muscle elements may increase their width during shortening with resultant increase in thickness and a decrease in longitudinal tension. It has been reported that vascular smooth muscle is arranged in a more helical fashion in central large arteries and in a more radial fashion in smaller peripheral vessels (10). Differences in arrangement of smooth muscle elements from animal to animal may thus account for the difference in longitudinal tension developing during constrictor responses in these studies.

Summary. Longitudinal tensions were recorded from *in situ* isolated segments of dog anterior tibial arteries during vasoconstrictor responses. Two factors appeared to be responsible for longitudinal tensions occurring during vasoconstrictor responses. One was due to changes in intraluminal pressure and appeared to be the dominant factor. Increases in intraluminal pressures produced decreases in longitudinal tensions, indicating that the vessel segments were elongated. Thus, increases in intraluminal pressure passively stretched the blood vessel walls so that increases in both the circumferential and longitudinal axes of the vessel segment occurred. These findings agree with similar findings reported earlier (4, 7, 8). The other factor was apparently due to changes in longitudinal tension produced by vascular smooth muscle contraction. In some cases longitudinal tension changes were positive, in others they were negative. Increases in longitudinal tension were probably the result of contractions of helical smooth muscle elements. Decreases in tension may have been due to displacement of tissue parallel to the long axis of the vessel as smooth muscle elements contracted.

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OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 183-185 (1976)

Adjuvant Arthritis in T Lymphocyte Depleted Rats[1] (39356)

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ivant arthritis in rats, induced by in- of oil and tubercle bacilli, is me- through lymphoid cells. Passive r into syngenic recipients of cells 'mph nodes of rats with adjuvant dis- ansferred the disease (1, 2). Simi- ransfer of the disease followed infu- thoracic duct lymphocytes from sen- donors (3). From these observations, ied likely that the T lymphocyte was portant cell in induction of adjuvant s, and the disease has been consid- some type of delayed sensitivity re- in which T cell function is crucial. t this general hypothesis, however, finding of Arnason *et al.* that neo- ymectomy markedly suppressed tu- n skin sensitivity but had little if any n adjuvant arthritis (4). Also, recent have indicated definitely that rat c duct lymph contains both T lym- es and long-lived recirculating B cells Finally, Lennon and Byrd (7) re- reported adjuvant arthritis occurring ttempts to develop allergic encepha- itis (EAE) in rats, but with arthritis ping *only* in neonatally thymecto- animals. In those experiments, adju- thritis developed in rats with lympho- evidence of depressed T cell func- nd poor humoral antibody responses lin protein used to produce EAE.

experiments were designed to study nt arthritis in a highly susceptible rat in animals made specifically T cell nt but with normal B cells.

rials and methods. Male and female Evans rats, inbred at the University of ky by Dr. Katherine Sydnor, were these experiments, because studies laboratory revealed that this strain regularly developed severe adjuvant disease. T lymphocytes were defined by their unusual affinity for uptake of tritiated uridine by methods described in previous studies from this laboratory (6).

T depleted rats were prepared by the method of Howard and colleagues (8), somewhat modified as described below. At age 7 or 8 days, a time when thymocytes in rats have migrated to nonthymic sites, thymectomy was performed using hypothermic (crushed ice) anesthesia, as described for newborn rats (9). The animals were returned to their mothers and grew normally. At about 3 months of age, blood was drawn for T cell determinations by [^{3}H]uridine incubation, and 1 week later the rats received 850-rad total body irradiation from a cobalt-60 source. Within a few hours of otherwise lethal irradiation for rats of this age, hematopoietic cells and B lymphocytes were repopulated by intravenous injection of 10^7 viable bone marrow cells from a syngenic donor that had been depleted of T lymphocytes by 5 days of thoracic duct drainage.

Blood counts and [^{3}H]uridine studies were followed for 6 weeks until the total lymphocyte count and T and B populations were stable. At that point, the left hind paw of 14 rats was injected with 0.1 ml of Freunds complete adjuvant (3 mg tubercle bacilli/ml). Normal Long-Evans rats of the same age and sex (about 4½ months) were injected at the same time. Every other day thereafter, the animals were observed for the onset of arthritis and the severity quantitated by a cumulative severity index using a slight modification of the scoring technique described by Pearson (10).

At the end of the experiment, when the arthritis was inactive, rats were again studied for T and B lymphocytes in blood, and a few animals underwent thoracic duct drainage for evaluation of quantitative and qualitative changes in cells of their thoracic duct lymph.

Results. A. Studies of the extent of T cell

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depletion. Unlike neonatal thymectomy which leads to near absence of T cells with runting disease and severe defects in cellular immune reaction, these animals were less severely depleted and appeared grossly normal. In Table I blood small lymphocytes and total T and B cells are shown for normal Long-Evans rats, and for the experimental group 3 months after thymectomy and before irradiation, at 6 weeks after irradiation and bone marrow repopulation, and again 8 weeks later at the end of the arthritis experiment. In looking closely at the data in Table I, T cell depletion by thymectomy at 8 days of age was only slightly enhanced by thymectomy + irradiation + reconstitution with T depleted bone marrow. B cells remained relatively intact in all groups. At least 22 weeks after thymectomy, irradiation, reconstitution, and arthritis, T cells remained low.

Thoracic duct lymph drainage, performed 16 weeks after thymectomy, irradiation, and marrow replacement in a few rats, confirmed the significant and persistent T lymphocyte depletion. Total cell count declined from about 12,000/mm^3 to about 3000/mm^3, and percentage T ([^{3}H]uridine-labeled small lymphocytes) from 70 to about 8%.

B. Severity and incidence of adjuvant arthritis. All 12 T-depleted rats and their 10 controls developed some degree of arthritis, and the severity index is plotted in Fig. 1. At all points, the T-deficient rats had less severe arthritis. Statistical validity was checked for the Day 20 scores, and the differences were significant ($P = < 0.05$).

Tuberculin sensitivity was checked in all rats on Day 14 by intradermal injection of 0.1 ml of P.P.D. (720 μg/ml) in saline, and 8/10 control animals and 6/12 T-depleted rats reacted. In those rats who did develop tuberculin sensitivity, the size of the reaction 48 h after test was similar in both groups (12 and 11 mm erythema, respectively).

Discussion. Adjuvant arthritis is as unique to rats as rheumatoid arthritis is to man, and continued study of the immunological mechanisms involved in the rat model may prove helpful in understanding the human disease. In this study, highly susceptible Long-Evans rats were made T cell deficient by thymectomy at about the end of the first week of life, and by later irradiation and reconstitution with small numbers of bone marrow cells from syngenic donors depleted of marrow T cells. As evidenced by avid [^{3}H]uridine uptake and radioautography, a label quite specific for T cells in the

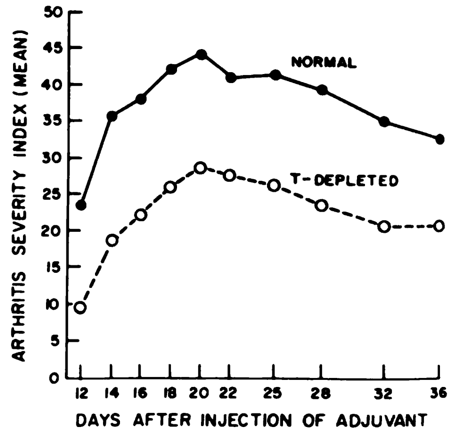

FIG. 1. Arthritis severity index of normal and T lymphocyte depleted Long-Evans rats. All rats in both groups (10 normal and 12 T-depleted) developed arthritis.

TABLE I. LYMPHOCYTE STATUS (T + B CELLS) OF RATS AT DIFFERENT STEPS IN THE STUDY.

Rat status	Number	Total small lymphs per mm^3 (× 10^3)	Total T per mm^3 (× 10^3)	Total B per mm^3 (× 10^3)
Normal	6	3.6 ± 0.8	1.3 ± 0.4	2.4 ± 0.6
Normal, 6 weeks post adjuvant	8	3.6 ± 0.9	1.8 ± 0.5	1.6 ± 0.5
Normal, 16 weeks post adjuvant	6	3.8 ± 1.1	1.9 ± 0.7	1.9 ± 0.5
Post Tx[a], 12 weeks	10	1.8 ± 0.5	0.4 ± 0.2	1.4 ± 0.5
Post Tx, 6 weeks post irrad.[b] + recon.[c]	6	1.6 ± 0.9	0.2 ± 0.05	1.4 ± 0.6
Post Tx, irrad. + recon., 6 weeks post adjuvant	8	1.3 ± 0.5	0.2 ± 0.1	1.2 ± 0.6
Post Tx, irrad. + recon., 16 weeks post adjuvant	6	1.7 ± 1.0	0.3 ± 0.2	1.4 ± 0.9

[a] Tx = thymectomy.

[b] irrad. = 850 rad irradiation.

[c] recon. = Reconstitution with 10^7 T-depleted marrow cells.

such rats maintained about 20-30%
nal T cells during the 7-8 months of
All experimental animals developed
nt arthritis, but with modest decrease
rity of the disease when compared to
rats.

ion and Byrd (7) tumbled onto a sys-
which T cell depletion actually en-
adjuvant arthritis. Their methods
ifferent; a relatively resistant Lewis
of rats, slightly different quantity of
e bacilli in the adjuvant, presence of
basic protein in the Freund's adju-
nd booster adjuvant in the form of
is vaccine. Taken together with our
, however, it is evident that the im-
ogical mechanisms for adjuvant ar-
are complicated. Paterson (11) has
ed the role of adjuvants in allergic
alomyelitis (EAE), and also con-
that mechanisms are much more
x than simply a generalized enhance-
of T cell function. He suggests that
adjuvants may work by enhancing
ation of the target organ, and others
arkedly influence different functional
ses of T lymphocytes. Lennon and
7) conclude that their results might be
ed by immune complexes formed in
a of antigen excess. Continued work
noral antibody production and spe-
lymphocyte subpopulations may help
the growing complexity of the
ism by which adjuvants produce a
toidlike arthritis specifically in rats.

nary. Markedly T lymphocyte de-
rats were prepared by thymectomy, irradiation, and repopulation by bone marrow hematopoietic and lymphoid cells. Such rats had persistent T lymphopenia of about 20% of normal. When T depleted and normal rats were injected with adjuvant, all animals developed arthritis but with slightly less severity in the T depleted animals. Such experiments, and other observations, suggest a complex immunological mechanism in the pathogenesis of adjuvant arthritis in the rat.

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Therapeutic Activity of Pretazettine, a Narcissus Alkaloid on Rauscher Leukemia: Comparison with Tazettine and Streptonigrin[1] (39357)

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One of the narcissus alkaloids (residual alkaloid A-2) has been shown to be therapeutically effective against advanced Rauscher leukemia in mice (1-3). The alkaloid inhibits the growth of Rauscher virus (2), a viral reverse transcriptase (4), and cellular protein synthesis (5). The alkaloid was identified as pretazettine, an unstable free base (3). We have now succeeded to isolate enough amounts of pretazettine hydrochloride via the picrate (6). We now report the anti-Rauscher leukemic activity of pretazettine HCl (PTZ) in comparison with tazettine (TZ) and streptonigrin (SN), one of the few inhibitors of reverse transcriptase, effective *in vivo* against Rauscher leukemia in mice (7).

Materials and methods. Preparation of agents. The methods of isolation of PTZ and TZ from the bulbs of *Narcissus tazetta L* have been described (6). The antibiotic SN was obtained from Dr. Chirigos of NIH. PTZ and SN were dissolved in distilled water and TZ was dissolved in water slightly acidified with HCl (pH 5) before use.

Cell cultures. NIH/3T3 and BALB/3T3 mouse fibroblast cell lines, obtained from Dr. Aaronson of NIH and cultured in MEM medium with 10% fetal calf serum for several months in this laboratory, were used for the *in vitro* antiviral testing of the agents. XC cells, supplied by Dr. Hackett of Naval Biomed. Res. Lab., Oakland, Calif., and kept in MEM medium with 5% fetal calf serum, were used as detectors of Rauscher virus.

System of Rauscher viral leukemia and chemotherapy. An inoculum (0.2 ml) of a 1:40 dilution of leukemic plasma was injected intraperitoneally (ip) into 8-10-week-old BALB/c inbred mice, either male or female. Treatment with optimal dose of the agents was started subcutaneously (sc) 2 to 3 weeks after infection when 70 to 100% of the mice showed clearly palpable spleens. In some experiments, the treatment was started earlier. Agents were injected every other day for several weeks. The dose and the periods were chosen to avoid any apparent toxicity, as indicated by general weakness, ruffled hair, weak motions, or marked loss of body weight.

In vitro test for antiviral activity. NIH/3T3 or BALB/3T3 cells, freshly trypsinized and suspended in 5 ml of culture medium (ca. 2 × 10^4 cells/ml) in Falcon plastic flasks (25-cm growth area) were inoculated with 0.5 ml of 1:10 dilution of leukemic plasma (ca. 10^5 XC syncytial forming units). The cells were inoculated at 37° for 3 days with daily medium changes; then various doses of the agents in 0.1 ml of distilled water were added (on Day 3) and incubated for 2 more days. The culture medium was then collected (on Day 5) for the titration of virus yield and the cultures were overlaid with 5 × 10^5 XC cells on 5 ml of MEM medium with 5% calf serum. The number of syncytial giant cells per microscopic field under 40× magnification one day later (on Day 6) was counted. For the titration of the virus yield in the harvests, NIH/3T3 cells were used. The cells (10^4) freshly suspended in 0.5 ml of medium were inoculated with 0.1 ml of a 10-fold dilution of the harvests and incubated at 37° for 9 days with medium changes of every other day, then 5 × 10^4 XC cells in 1 ml were overlaid and incubated 1 more day. The syncytial cells were detected by the modified method of XC assay of Rowe *et al.* (8). Three tubes were used for each dilution. The virus titer was expressed by a median tissue culture infectious dose ($TCID_{50}$)/ml.

Results. Effect of pretazettine HCl, tazettine, and streptonigrin on survival of mice

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with established Rauscher leukemia. Three initial experiments with the purified residual alkaloid, PTZ, have been performed to confirm our previous experiments (1-3) with the crude alkaloid fractions. Figure 1 or 2 shows the dynamic course of the leukemic disease which the treatment was started on the early (Day 7) or on the advanced (on Day 25) stage after the ip inoculation of Rauscher virus. The median survival time (MST) of the PTZ-treated group was 81 days (controls: 41 days) in Fig. 1, or 72 days (controls: 47 days) in Fig. 2. That is, the continuous administration of PTZ significantly increased ($P < 0.01$) the life span of the leukemic mice. As shown in Fig. 3, PTZ was still effective against the leukemia which was initiated by the intracerebral inoculation of the virus. While pretazettine (Fig. 4) is stable in acidic pH, it is unstable in basic pH and gradually rearranged to tazettine (Fig. 4) (9, 10) on standing. Since it is likely that PTZ will be converted to TZ in the body, it is of interest to know whether TZ has the biological potency. It is also of interest to compare PTZ with streptonigrin, an antibiotic which is one of the few inhibitors of viral reverse transcriptase, possessing therapeutic activity against Rauscher viral leukemia (7). Table I shows the results of six comparative experiments with TZ and three with SN. It was found that TZ was not significantly active (the mean prolongation effect of MST = 22%; $P > 0.05$), while SN demonstrated almost the same activity as that of PTZ. Although it is active, SN

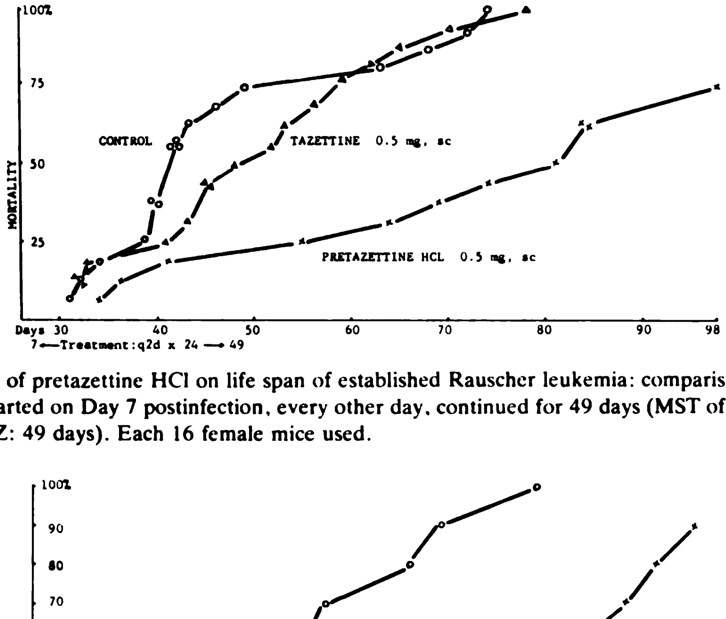


FIG. 1. Effect of pretazettine HCl on life span of established Rauscher leukemia: comparison with tazettine. Treatment was started on Day 7 postinfection, every other day, continued for 49 days (MST of control: 41 days; PTZ: 81 days; TZ: 49 days). Each 16 female mice used.

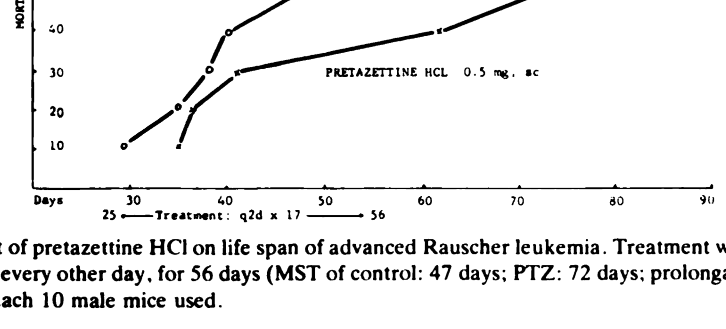


FIG. 2. Effect of pretazettine HCl on life span of advanced Rauscher leukemia. Treatment was started on Day 25 postinfection, every other day, for 56 days (MST of control: 47 days; PTZ: 72 days; prolongation of MST over control: 53%). Each 10 male mice used.

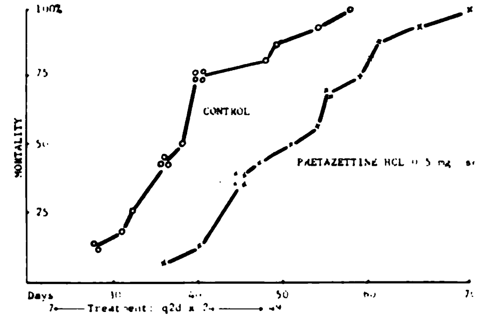
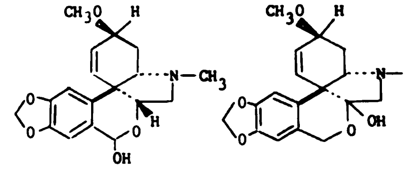


FIG. 3. Effect of pretazettine HCl on life span of established Rauscher leukemia by ic route-infection. Treatment was started 7 days after intracerebral inoculation of virus, every other day, continued for 49 days, total 24 injections (MST of control: 38 days; PTZ: 51 days; prolongation of MST over control: 34%, $P < 0.05$). Each 16 female mice used.

showed unfavorable delayed side effects such as local skin-muscle necrosis, ruffled hair, weak motions, and loss of body weight within 2 to 3 weeks, so further administration was not possible, while PTZ administration could be continued for over 100 days (E15) without apparent toxicity. In E16, the mice were inoculated with a washed leu-



Pretazettine Tazettine

FIGURE 4

TABLE I. EFFECT OF PRETAZETTINE HCl ON SURVIVAL OF MICE WITH ESTABLISHED RAUSCHER LEUKEMIA: COMPARISON WITH TAZETTINE AND STREPTONIGRIN.

Expt. no.	Agent	Dose (mg/kg)	Times and period of treatment (days)	Median survival time (MST) (day and range)	Prolong. of MST over control (%)	P
E11	Control (each 20 female)			29 (24-46)[b]		
	Pretazettine	25	10 (18-37)[a]	45 (31-78)	55	<0.01
	Tazettine	25	10 (18-37)	37 (23-49)	28	<0.05
E12	Control (each 20 male)			53 (27-74)	55	<0.01
	Pretazettine	25	10 (23-42)	82 (32-107)	19	>0.05
	Tazettine	25	10 (23-42)	63 (30-78)		
E13	Control (each 20 female)			44 (28-50)		
	Pretazettine	25	17 (17-39)	109 (32-141)	148	<0.01
	Tazettine	25	17 (17-39)	53 (28-62)	25	<0.05
E14	Control (each 20 male)			43 (29-55)		
	Pretazettine	25	21 (19-56)	76 (32-85)	77	<0.01
	Tazettine	25	21 (19-56)	58 (31-62)	35	<0.05
E15	Control (each 20 female)			40 (19-62)		
	Pretazettine	25	56 (5-108)	98 (40-135)	145	<0.01
	Tazettine	25	56 (5-108)	49 (19-68)	23	>0.05
E16	Control (each 20 female)			29 (23-31)		
	Pretazettine	25	20 (12-53)	74 (30-77)	155	<0.01
	Tazettine	25	8 (12-27)	27 (23-34)	0	
E17	Control (each 10 female)			40 (21-49)		
	Pretazettine	25	7 (16-30)	61 (27-76)	53	<0.01
	Streptonigrin	0.05	7 (16-30)	63 (30-68)	58	<0.01
	Streptonigrin	0.025	12 (16-40)	48 (24-55)	20	>0.05
E18	Control (each 10 female)			44 (20-63)		
	Pretazettine	25	10 (18-37)	69 (33-89)	57	<0.01
	Streptonigrin	0.05	10 (18-37)	67 (35-81)	52	<0.01
	Streptonigrin	0.025	14 (18-45)	53 (32-64)	18	>0.05
E19	Control (each 20 male)			48 (21-78)		
	Pretazettine	25	10 (18-37)	79 (25-92)	65	<0.01
	Streptonigrin	0.05	10 (18-37)	85 (31-89)	77	<0.01
	Streptonigrin	0.025	14 (18-45)	60 (27-75)	25	<0.05

[a] Treatment with the optimal dose of each agent was started on Day 18 and continued every other day until Day 37, total 10 injections. Only in E13, five daily injections per week were done.

[b] Day of the first death to day of the last death during the observation periods of 100–150 days.

c whole blood (0.2 ml of 1:20 dilution,
reshly harvested, which contained at
10^5 levels of leukemic cells. PTZ was
therapeutically effective against
cher leukemia established by trans-
ed leukemia cells.

totoxicity test of agents against NIH/
cells. Before testing the antiviral activ-
f the agents, the maximum nontoxic
(MNTD) for the long-term and short-
cultivations of the cells were deter-
d. It was found that the cells could be
nuously passaged at a slightly reduced
of growth in the presence of PTZ at the
mum concentration of 50 ng/ml, TZ at
(5000 ng)/ml, and SN at 0.5 ng/ml of
um. Doses higher than these were in-
ory to the growth of cells and decreased
ell populations gradually, and finally
l not maintain the cultures. When the
of PTZ or TZ was increased up to 5 or
g/ml, respectively, the cells which had
making confluent or subconfluent
layer became static within 2 days of
ation, recognized by reduced acid pro-
on, skinny fibroblastic forms, but still
y attached to the wall. The majority of
ells quickly recovered when the agents
removed from the medium 2 days
. When the dose of SN was increased up
0 ng/ml, the cells became apparently
within 2 days. The cells were affected
e agent at the range of 5 to 100 ng/ml
ut morphological changes, but many
gradually detached from the wall dur-
he further incubation, after removing
gent from the medium. Thus, the static
s of the cells induced by PTZ (0.2 to 5
l) or TZ (20 to 100 μg/ml) was reversi-
hile the same status by SN (5 to 100
l) was irreversible. Any higher levels of
gents (PTZ 10 μg, TZ 200 μg, and SN
g/ml) destroyed the monolayers of the
and over 50% of the cells were de-
d from the wall within 2 days. After
ing their cytotoxic doses, the antiviral
ity of these agents were tested at var-
doses up to MNTD for 2 days incuba-
tion with the agents. The virus growth in the cells was directly estimated by XC cells overlaid on the cultures on Day 5. The three agents showed clear inhibitory effect on the growth of virus at the cell-static doses. PTZ possessed a wider effective range (0.5 to 5 μg/ml) compared with TZ (25 to 100 μg) and SN (50 to 100 ng). Table III shows the antiviral activity of these agents at the optimal dose both in NIH and BALB/3T3 cells, estimated by the virus titration of the medium in addition to the direct XC overlay. PTZ suppressed the virus growth 2 log below the level of controls, while TZ and SN one log.

ect of PTZ, TZ, and SN on Rauscher
growth in 3T3 cells. Table II shows the
s using NIH/3T3 cells infected with
rus 3 days prior to the 2 day-treatment

Discussion. The free base pretazettine (residual alkaloid A-3, amorphous powder) was reported to be unstable in water above pH 7 and gradually rearranged to the stable isomer TZ even on standing at room temperature (9, 10). The hydrochloride of the pretazettine (PTZ), a stable form in water, is now quantitatively prepared by a modi-

TABLE II. EFFECT OF PTZ, TZ, AND SN ON RAUSCHER VIRUS GROWTH IN NIH/3T3 CELLS.[a]

	Number of syncitial cells			
Agent and dose/ml	Expt. 1	Expt. 2	Expt. 3	Mean (%)
Control (no agent)	65	63	20	100
Pretazettine HCl (μg)				
5	0	0		0
2.5	0	0	2	1
1.0	2	5		6
0.5	2	4	6	8
0.25	7	9		13
0.1	25		9	40
0.05	58	51	22	102
Tazettine (μg)				
100	3		0	2
50	6	8	5	13
25	28	35		49
10	62	70	18	101
5	72	68		110
Streptonigrin (ng)				
100	10	2	1	9
50	30	15		35
20	68	70	19	107
10	62	59		94
5	65		25	106

[a] Treatment with various nontoxic dose of each agent was started 3 days after the virus infection and continued for 2 days, then XC cells were overlaid. One day later, the number of syncitial giant cells per a microscopic field under 40× magnification was counted. The number is the average of 5 fields counted.

TABLE III. EFFECT OF PTZ, TZ, AND SN ON RAUSCHER VIRUS GROWTH IN NIH AND BALB/3T3 CELLS.[a]

Agent and dose/ml	Virus yield[b] 10 log $TCID_{50}$/ml	Syncitial cell[c] formation (%)
NIH/3T3 cells		
Control	5.5	100
PTZ 1 μg	3.5	9
TZ 50 μg	4.2	15
SN 50 ng	4.2	32
BALB/3T3		
Control	4.5	100
PTZ 1 μg	2.2	1
TZ 50 μg	3.5	21
SN 50 ng	3.2	19

[a] Treatment with the optimal dose was started 3 days after infection and continued for 2 days, then the medium was harvested for the virus titration and XC cells were overlaid.

[b] The virus yield in the harvests were titrated using NIH/3T3 cells as described in Methods.

[c] The number of syncitial cells per a microscopic field (40×) was counted 1 day after XC overlay.

fied method of Wildman (6) in this laboratory. It was of interest to know whether TZ, a more stable isomer of pretazettine, is still active. It was found that TZ was not significantly effective against Rauscher leukemia in mice and it was needed at 50 to 100 times greater dose of PTZ to inhibit the virus growth *in vitro* (Tables II and III), while PTZ was therapeutically effective as same as residual alkaloid A-2 previously reported (3). At 60 μg/ml, PTZ was inhibitory (50%) to reverse transcriptase of avian myeloblastosis virus (communication from Dr. Chirigos of NIH) and we have confirmed the inhibitory activity on the reverse transcriptase of Rauscher virus at concentrations of 50 to 100 μg/ml (unpublished data), while TZ showed no activity on the enzymes of both viruses. Recently, at 10 μg/ml, PTZ has been found to inhibit the cellular protein synthesis without affecting the syntheses of DNA and RNA in KB, P388, and Ehrlich ascites cells in this laboratory (5), and in HeLa and Krebs II cells (communication from Dr. D. Vazques, Inst. Cellular Biology, Madrid, Spain) but TZ showed no inhibition. Such findings mentioned above suggest that the PTZ is the active form and TZ is an inert isomer. The MNTD of SN for the long-term cultivation of NIH/3T3 cells (0.5 ng/ml) was almost equivalent to the MNTD of the plating efficiency of cell colony formation methods reported by other laboratories: 0.16 to 0.33 ng/ml by Price *et al.* (11) and 0.5 ng/ml by Woods *et al.* (12). Although SN at such a low concentration which did not suppress the cell growth was reported to inhibit the *in vitro* transformation of high passage rat embryo cells by 3-methylcholanthrene (3CM) (11), it was not inhibitory to Rauscher virus growth in NIH/3T3 cells. In our system, at least 100 times more concentration (50 ng/ml) of SN was required to stop the on-going virus growth initiated 3 days previously, and such a high concentration of SN made the cells apparently static but actually irreversibly destroyed the growth viability. On the contrary, PTZ at a low concentration which was not inhibitory to cell growth (0.05 μg/ml) could not prevent the transformation of the rat cells by 3CM (communication from Dr. Price of Microbiological Associates, Maryland); however, at 10 times higher concentration (0.5 μg/ml) PTZ made the cells reversibly static and was inhibitory to the on-going growth of Rauscher virus in 3T3 cells.

Summary. The therapeutic activity of narcissus alkaloid pretazettine HCl (PTZ) on established Rauscher leukemia has been demonstrated and compared with the isomer tazettine (TZ) and an antibiotc, streptonigrin (SN). PTZ and SN showed remarkable prolongation effect on the life span of the leukemic mice and the antiviral activity has been confirmed in mouse 3T3 cells infected with Rauscher virus. TZ showed no significant activity in the leukemic mice and was inhibitory to the virus growth in the cells at much higher doses than PTZ. It is suggested that the stereochemical rearrangement from PTZ to TZ inactivates the biological activity of PTZ.

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Zinc Metabolism and Homeostasis in Rats Fed a Wide Range of High Dietary Zinc Levels (39358)

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The essentiality of zinc and its importance in animal and human nutrition are well known. However, many key aspects of zinc metabolism and homeostasis remain to be established (1). In studies with calves (2-5), adding 600 ppm supplemental zinc to a practical diet drastically increased zinc in certain tissues, especially liver, kidney, and pancreas, indicating a breakdown of zinc homeostasis. With rats the same diet did not materially affect the zinc content of liver and kidneys suggesting that homeostatic control mechanisms for zinc are much more effective in this species than in calves (6).

In this study effects of a wide range of dietary zinc levels (38 to 8438 ppm) on zinc and ^{65}Zn metabolism and homeostasis in rats were investigated.

Materials and methods. Sixty-eight male Cherokee S-D Albino rats, initially weighing 100–120 g and being about 35 days of age, were fed a practical corn–soybean diet (2) containing 38 ppm zinc, or the same diet supplemented with 1200, 2400, 3600, 4800, 6000, 7200, or 8400 ppm zinc as ZnO for 21 days. The rats, maintained individually in stainless steel cages, were fed and given distilled water *ad libitum*. The rats were observed routinely throughout the experiment for clinical symptoms of toxicity such as skin lesions, diarrhea, muscular incoordination, and reduced feed intake.

Fourteen days after initiation of dietary treatments, each rat was anesthetized and given by gavage an oral tracer dose of 29.8 μCi $^{65}ZnCl_2$ in acetate buffer (pH 4.7, sp act of 4.25 mCi/mg Zn). Total fecal collections were made for 7 days after dosing, at which time the rats were anesthetized with diethyl ether and sacrificed by exsanguination. Blood, heart, liver, kidneys, round muscle, and tibia samples were taken.

Zinc in feed, feces, and tissues was determined by atomic absorption spectroscopy, after nitric–perchloric–sulfuric acid wet ashing of samples (7). ^{65}Zn activity was determined with an automated gamma ray test tube changer system with a NaI (T1) well crystal.[1] Because of nonhomogeneity of variances (due to large differences in mean values), the data were transformed to common logarithms before testing by analysis of variance and Duncan's Multiple Range Test procedures (8).

Results. The high zinc diets did not affect weight gains or feed consumption or cause clinical toxicity symptoms. Average daily feed consumption for controls and those receiving 8400 ppm added zinc were 18 and 19 g, respectively, while weight gains were 3.5 and 3.3 g per day, respectively.

Stable zinc excreted in the feces increased linearly with each additional increment of dietary zinc (Fig. 1A). In contrast, total fecal excretion of ^{65}Zn increased sharply from 65% of the dose in controls to 86% of the dose in rats given 1200 ppm added zinc (Fig. 1B). Further additions of dietary zinc up to 8400 ppm had little effect of fecal ^{65}Zn excretion (Fig. 1B).

Liver, kidney, and tibia stable zinc increased ($P < 0.05$) with added dietary zinc up to 2400 ppm (Table I). Although data were variable, a plateau in zinc concentration of these tissues was evident from the 2400 to about 7200 ppm added zinc. Further increases of zinc in liver, kidney, and tibia occurred when supplemental zinc was increased from 7200 to 8400 ppm. Muscle and heart zinc were not appreciably affected by any of the dietary treatments.

The high ^{65}Zn content in tissues of control rats (38 ppm dietary Zn) (Table II) relative to those given supplemental zinc indicates much more labile zinc. Although only a small fraction of a kilogram of tissues is

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[1] Model 709, Baird Atomic, Inc., Cambridge, Mass.

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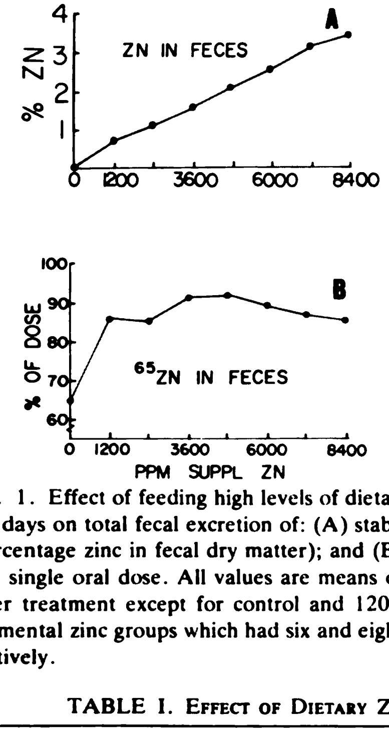

FIG. 1. Effect of feeding high levels of dietary zinc for 21 days on total fecal excretion of: (A) stable zinc (as percentage zinc in fecal dry matter); and (B) ^{65}Zn from a single oral dose. All values are means of nine rats per treatment except for control and 1200 ppm supplemental zinc groups which had six and eight rats, respectively.

involved, the ^{65}Zn data are presented per kilogram to obtain convenient sized numbers (Table II).

Feeding 1200 ppm supplemental zinc sharply reduced ^{65}Zn retention in all tissues but further added zinc to 8400 had much less effect (Table II). Muscle and heart ^{65}Zn declined significantly when added zinc was increased from 1200 to 8400 ppm. In contrast, bone ^{65}Zn increased when added dietary zinc was advanced from 3600 to 6000 ppm and remained fairly constant with still higher zinc intakes (Table II).

Discussion. Homeostatic control mechanisms regulate tissue concentration of essential elements with different degrees of effectiveness in animals consuming varying intakes (1, 9, 10). In calves, these control mechanisms for zinc operate through changes in absorption, endogenous fecal excretion, and tissue turnover rates (1, 9). In this study, with young growing rats, linear

TABLE I. EFFECT OF DIETARY ZINC LEVEL ON STABLE ZINC IN SELECTED TISSUES.^{a}

Dietary zinc added (ppm)	Tissues (ppm zinc, dry tissue)				
	Liver	Kidneys	Heart	Tibia	Muscle (round)
Control^{a}	92 ± 3^{b}	96 ± 9^{b}	71 ± 5^{b}	246 ± 31^{b}	43 ± 2^{b}
1200	170 ± 30^{bc}	145 ± 21^{bc}	69 ± 2^{b}	456 ± 85^{bc}	46 ± 5^{b}
2400	242 ± 36^{cd}	237 ± 43^{cd}	71 ± 3^{b}	866 ± 161^{cd}	60 ± 9^{b}
3600	201 ± 42^{c}	220 ± 45^{cd}	73 ± 5^{b}	631 ± 160^{bc}	52 ± 6^{b}
4800	184 ± 40^{bc}	229 ± 52^{cd}	75 ± 3^{b}	748 ± 208^{c}	48 ± 5^{b}
6000	210 ± 50^{c}	209 ± 38^{cd}	78 ± 3^{b}	685 ± 165^{c}	43 ± 3^{b}
7200	243 ± 27^{cd}	238 ± 41de	73 ± 3^{b}	904 ± 153^{cd}	48 ± 5^{b}
8400	379 ± 41^{d}	408 ± 48^{e}	94 ± 6^{c}	1407 ± 204^{d}	51 ± 7^{b}

[a] Values are mean ± SE for nine rats except control and 1200 ppm zinc supplemental groups which has six and eight rats, respectively. Control diet had 38 ppm zinc.

[b,c,d,e] Values in the same vertical column not followed by the same letter are significantly different ($P < 0.05$) as determined by analyses of variance of data transformed to common logarithms.

TABLE II. TISSUE ^{65}ZN DISTRIBUTION AS AFFECTED BY DIETARY ZINC.^{a}

Dietary zinc added (ppm)	Tissues (% of dose/kg fresh tissue)					
	Liver	Kidneys	Heart	Tibia	Muscle (Round)	Blood
Control^{a}	192.0 ± 6.4^{b}	145.9 ± 7.0^{b}	117.3 ± 4.1^{b}	525.4 ± 54.3^{b}	78.2 ± 4.7^{b}	38.6 ± 2.0^{b}
1200	7.7 ± 0.9^{c}	5.4 ± 0.6^{cd}	4.8 ± 0.6^{c}	25.9 ± 3.3^{c}	3.5 ± 0.6^{c}	1.6 ± 0.2^{c}
2400	6.2 ± 1.2^{cd}	4.4 ± 0.8^{de}	2.8 ± 0.4^{cd}	29.3 ± 4.2^{c}	2.8 ± 0.5^{cd}	1.4 ± 0.3^{cd}
3600	4.4 ± 0.6^{d}	3.3 ± 0.4^{e}	1.9 ± 0.3^{def}	37.0 ± 5.6^{c}	2.1 ± 0.2^{cde}	0.8 ± 0.1^{e}
4800	5.4 ± 0.5^{cd}	4.2 ± 0.4^{de}	1.6 ± 0.2^{ef}	57.3 ± 5.4^{d}	1.7 ± 0.1^{def}	0.8 ± 0.1^{e}
6000	8.1 ± 1.8^{c}	6.3 ± 1.3^{cd}	2.2 ± 0.5^{de}	97.2 ± 22.6^{c}	1.5 ± 0.5^{ef}	1.1 ± 0.3^{de}
7200	6.2 ± 0.5^{cd}	5.6 ± 0.6^{cd}	1.3 ± 0.2^{f}	91.2 ± 8.6^{c}	1.3 ± 0.1^{f}	0.9 ± 0.05^{de}
8400	6.3 ± 0.7^{cd}	6.3 ± 0.6^{c}	1.2 ± 0.2^{f}	92.8 ± 9.0^{c}	1.2 ± 0.1^{f}	1.0 ± 0.1^{de}

[a] Values are means ± SE for nine rats except control and 1200 ppm zinc supplemental groups which had six and eight rats, respectively. Control diet had 38 ppm zinc.

[b,c,d,e,f] Values in the same vertical column not followed by the same letter are significantly different ($P < 0.05$) as determined by analyses of variance of data transformed to common logarithms.

increases in fecal excretion of zinc with each increased in dietary zinc contributed substantially to homeostatic control. The decreased retention of stable zinc and the contrasting plateau of fecal ^{65}Zn retention indicates a more rapid zinc turnover rate and suggests higher endogenous losses when added dietary zinc was elevated above 1200 ppm (Fig. 1).

The sharp increases of stable zinc in the liver, kidney, and tibia with the highest zinc intake suggest some breakdown in zinc homeostatic control. The relatively constant heart and muscle zinc, regardless of dietary level, is consistent with previous rat (6) and calf (2–4) studies. Similarly, the plateau in zinc concentrations of liver, kidney, and tibia indicates good homeostatic control occurred in these tissues when supplemental dietary zinc was increased from 2400 to 7200 ppm. Failure of ^{65}Zn retention to decline in liver, kidney, and tibia when added zinc was elevated above 2400 ppm suggests that an increasing zinc turnover rate in these tissues is a factor in the homeostatic control. The increase in zinc turnover rates was less evident in muscle and heart ^{65}Zn data. Apparently, ^{65}Zn metabolism in bone is quite different from that of more biologically active tissues (10, 11).

In contrast to the 8400 ppm zinc required for major elevations of zinc in liver and kidney of rats, only 600 ppm added dietary zinc in calf diets causes much larger elevations in tissue zinc (2, 3). Thus, when high dietary zinc is fed, rats have much more effective homeostatic control of zinc levels in body tissues than calves.

Summary. Zinc metabolism and homeostasis were studied in young growing rats fed a 38 ppm zinc diet with added zinc levels ranging from 0 to 8400 ppm for 21 days. High dietary zinc did not cause toxicity symptoms. Stable zinc in feces increased linearly with dietary zinc intake but fecal ^{65}Zn, from a single oral dose, did not increase above the 1200 ppm dietary level. Stable zinc in liver, kidney, and tibia increased two to three times with 2400 ppm added zinc, but was not further elevated until 8400 ppm was fed. Stable zinc in muscle and heart was not affected appreciably by dietary zinc level. In all tissues, ^{65}Zn retention was drastically reduced with 1200 ppm added dietary zinc. Additional dietary zinc reduced ^{65}Zn in muscle and heart but had little effect on liver and kidney ^{65}Zn. The data indicate that rats have fairly effective homeostatic control mechanisms for tissue zinc below about 7200 ppm dietary zinc. Whereas, with dietary zinc up to about 1200 ppm, decreasing absorption is the main route of homeostatic control, above this level, more rapid zinc turnover rates and increasing endogenous zinc excretion appear to have major importance.

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rglycemic Activity of Some Non-Nitrosated Streptozotocin Analogs (39359)

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otozotocin, an antitumor and diabe- agent derived from glucose and 1- -1-nitrosourea (1) has been shown to e irreversible hyperglycemia in rats, nice, and rhesus monkeys (2–4). Dia- nset after intravenous streptozotocin a triphasic pattern with an early hy- emia (peaking at the second hour), d by hypoglycemia (most marked at venth hour), and finally, by perma- yperglycemia by the twenty-fourth

detailed mechanism for streptozoto- uced blood glucose changes is not tely understood. The work of Junod) assigns the permanent diabetogenic ty of streptozotocin to its β-cell cyto- ction. The early hyperglycemic phase ved to result from inhibition of β-cell secretion (6). Since streptozotocin poses rapidly at physiological pH to e diazomethane, a classic alkylating various authors (6–8) have suggested reptozotocin and related *N*-nitroso unds exert their actions via release of lkane. This possibility has been ex- in the following work by evaluating ects of non-nitrosated as well as nitro- nalogs of streptozotocin on rat blood .

rials and methods. Streptozotocin ndly supplied by Dr. G. C. Gerritsen, ojohn Company, Kalamazoo, Michi- he syntheses of (I)–(IV), the haloe- reptozotocin analogs, and of (V)- the *O*-acetylated *N*-aryl streptozoto- ilogs, have been reported elsewhere

Sprague-Dawley rats (Charles Laboratories) of 210–250 g body were used throughout. Prior to use, mals were kept at 30–31° and fed standard Wayne chows *ad libitum*. For measurement of effects on fasting blood glucose, rats previously fasted 48 hr were bled from the tail vein before dosing and 0.5, 1, 2, 3, 4, 5, 6, and 48 hr after intraperitoneal administration of drug or vehicle. After the sixth hour bleeding, all animals were fed rat chow *ad libitum*. Twenty-four and forty-eight hours after dosing, all animals were tested for glycosuria using Lilly Tes-Tape. Complete details of the methods for determining blood glucose and for rat oral glucose tolerance testing have been described elsewhere (10). After completion of the glucose tolerance tests, all animals were provided with food *ad libitum* and were tested for glycosuria 24–48 hr after administration of drug or vehicle. For the fasting blood glucose experiments and the glucose tolerance tests using streptozotocin and compounds I–IV, the compounds were administered within 5 min after dissolution in 0.01 *M* citrate buffer, pH 4.5. The non-nitrosated analogs (V–VIII) were given during glucose tolerance tests as a suspension in 0.5% methylcellulose.

Results. The effect of streptozotocin and the streptozotocin analogs on rat glucose tolerance is shown in Table I. Streptozotocin and all eight analogs in doses of 100 mg/kg ip produced significant increases in blood glucose compared to vehicle-treated animals. However, none of the analogs produced a sustained rise in blood glucose comparable to the parent compound. The non-nitrosated analogs (II) and (IV) produced greater decreases in glucose tolerance in doses of 100 mg/kg ip than their nitrosated counterparts (Analogs I and III). Only streptozotocin treated animals exhibited glycosuria 24–48 hr after dosing.

Several of the compounds were also studied for their ability to affect fasted rat blood glucose levels. The results are shown in Table II. Streptozotocin (100 mg/kg, ip) and

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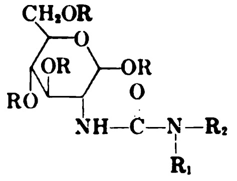
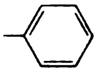
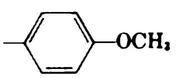
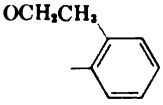
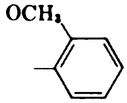


	R	R₁	R₂
Streptozotocin	H	$-N=O$	$-CH_3$
Analog (I)	H	$-N=O$	$-CH_2CH_2Cl$
(II)	H	$-H$	$-CH_2CH_2Cl$
(III)	$-\underset{\underset{O}{\|}}{C}-CH_3$	$-N=O$	$-CH_2CH_2Cl$
(IV)	$-\underset{\underset{O}{\|}}{C}-CH_3$	$-H$	$-CH_2CH_2Cl$
(V)	$-\underset{\underset{O}{\|}}{C}-CH_3$	$-H$	
(VI)	$-\underset{\underset{O}{\|}}{C}-CH_3$	$-H$	
(VII)	$-\underset{\underset{O}{\|}}{C}-CH_3$	$-H$	
(VIII)	$-\underset{\underset{O}{\|}}{C}-CH_3$	$-H$	

the nitrosated analog (I) (800 mg/kg, ip) produced the early hyperglycemic phase followed by a rapid lowering of blood glucose. All animals treated with (I) died between 7–24 hr after treatment presumably as the result of severe hypoglycemia. Streptozotocin also has been reported (11, 12) to produce lethalities 10 hr after administration of doses which produced a hypoglycemia effect comparable to that observed in the present studies with (I). In the present studies, the animals treated with 100 mg/kg ip of streptozotocin showed only modest hypoglycemia but had glycosuria 24–48 hr after dosing. The non-nitrosated analogs (II), (IV), (V), and (VII) also produced early hyperglycemia; however, neither the hypoglycemic phase nor permanent hyperglycemia and glycosuria were observed.

Discussion. Golden and co-workers (6) have demonstrated recently that streptozotocin can exert a direct inhibitory effect on glucose-induced insulin secretion by isolated islets of Langerhans. This indicates that a direct action on the β-cell, rather than a secondary liberation of glycogenolytic agents, such as epinephrine, is responsible for the early hyperglycemic phase after streptozotocin treatment. Since streptozotocin under various conditions has been found to be unstable, these authors postulated that the breakdown product diazoalkane was responsible for the early hyperglycemic phase. However, the present studies using streptozotocin analogs which cannot form diazoalkane breakdown products do not substantiate their conclusion. Not only did the nitroso analogs (I), (III), and streptozotocin produce early hyperglycemia but so did the non-nitrosated streptozotocin analogs. Moreover, the data suggest that the sugar moiety may be acting as more than just a nonspecific hydrophilic carrier for nitrosourea groups as has been suggested by numerous investigators (13–15) but may actually have a more direct effect.

Even though the non-nitrosated analog (II) produced more severe glucose intoler-

Expt. no.	Compound	N[b]	Blood glucose (mg/100 ml) at minutes after glucose X ± SEM						
			0	30	60	90	120	150	180
1	Vehicle	4	56 ± 1	105 ± 1	89 ± 1	83 ± 1	71 ± 1	66 ± 2	60 ± 2
	Streptozotocin	3	74 ± 3***	149 ± 4***	201 ± 5***	225 ± 7***	215 ± 4***	204 ± 3***	202 ± 9***
	(IV)	3	69 ± 4*	129 ± 1***	–	105 ± 2***	–	101 ± 1***	–
2	Vehicle	4	47 ± 2	135 ± 3	111 ± 5	101 ± 2	91 ± 4	95 ± 3	86 ± 3
	(I)	3	96 ± 5***	120 ± 5	123 ± 6	142 ± 5***	139 ± 6***	109 ± 2**	110 ± 3***
	(II)	3	89 ± 5***	208 ± 9***	213 ± 6***	178 ± 9***	140 ± 5***	123 ± 6**	117 ± 8**
3	Vehicle	4	44 ± 1	128 ± 2	113 ± 4	96 ± 3	90 ± 3	91 ± 2	–
	(III)	3	67 ± 2	128 ± 3	131 ± 2**	158 ± 4***	131 ± 4***	128 ± 5***	–
	(IV)	3	78 ± 3***	180 ± 5***	205 ± 4***	172 ± 3***	134 ± 6***	117 ± 2***	–
4	Vehicle	6	79 ± 4	170 ± 6	144 ± 4	121 ± 3	106 ± 4	95 ± 2	–
	(V)	3	86 ± 8	269 ± 8**	187 ± 10**	135 ± 4	118 ± 7	106 ± 7	–
	(VI)	3	89 ± 8	180 ± 16	189 ± 13**	154 ± 13*	124 ± 12	111 ± 5*	–
	(VII)	3	106 ± 5**	219 ± 12*	210 ± 7***	153 ± 5***	122 ± 2***	109 ± 3**	–
5	Vehicle	3	68 ± 2	172 ± 7	119 ± 7	90 ± 2	82 ± 2	78 ± 1	68 ± 2
	(VIII)	3	82 ± 3*	150 ± 9	164 ± 5*	128 ± 5*	100 ± 7	86 ± 2*	71 ± 2

[a] Compounds, 100 mg/kg, ip, or vehicle were given 30 min prior to oral administration of 1 g of glucose per kg body weight.

[b] Number of rats studied.

TABLE II. EFFECT OF STREPTOZOTOCIN AND STREPTOZOTOCIN ANALOGS ON 48-HR FASTED RAT BLOOD GLUCOSE.[a]

Treatment	Dose (mg/kg, ip)	Blood glucose (mg/100 ml) at minutes after glucose X̄ ± SEM								
		Initial	0.5	1	2	3	4	5	6	48
Vehicle	–	75 ± 6	81 ± 4	83 ± 3	69 ± 5	80 ± 4	87 ± 4	89 ± 3	88 ± 3	69 ± 3
(II)	200	69 ± 3	164 ± 10***	136 ± 7***	135 ± 5***	136 ± 6***	127 ± 7**	139 ± 8**	115 ± 4**	69 ± 6
	100	72 ± 3	136 ± 7***	137 ± 2***	108 ± 2***	112 ± 6**	105 ± 3**	101 ± 11	102 ± 8	86 ± 4*
(IV)	200	67 ± 1	122 ± 4***	132 ± 5***	131 ± 7***	136 ± 6***	143 ± 5***	123 ± 2***	113 ± 1***	81 ± 2*
	100	69 ± 1	125 ± 1***	135 ± 3***	139 ± 6***	114 ± 1***	102 ± 2**	105 ± 1**	121 ± 4***	88 ± 4*
Streptozotcin	100	79 ± 3	127 ± 2***	151 ± 3***	210 ± 2***	270 ± 4**	318 ± 9***	204 ± 7***	82 ± 5	484 ± 2***
Vehicle	–	67 ± 2	69 ± 1	77 ± 2	67 ± 2	65 ± 2	67 ± 2	66 ± 2	71 ± 4	123 ± 5
(I)	800	60 ± 2	94 ± 5**	114 ± 1***	156 ± 6***	79 ± 3*	48 ± 3**	25 ± 2**	23 ± 3***	–[b]
(II)	800	62 ± 1	111 ± 2***	140 ± 3**	158 ± 4***	158 ± 5***	130 ± 1***	128 ± 3***	118 ± 3***	129 ± 9
(V)	400	61 ± 2	80 ± 1**	87 ± 3**	85 ± 4*	80 ± 3*	84 ± 3**	86 ± 6*	101 ± 2***	111 ± 7
(VII)	365	62 ± 1	80 ± 5	85 ± 2***	88 ± 5**	102 ± 2***	97 ± 4***	92 ± 7**	81 ± 2*	118 ± 2

[a] Four rats per group. Significance compared to controls determined by Student's *t* test, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

[b] Lethal, 4/4.

ance at 100 mg/kg ip (Table I) and at least as great a fasting hyperglycemia in doses of 800 mg/kg ip (Table II) as its corresponding nitrosated analog (I), only (I) induced a rapid phase of blood glucose lowering similar to streptozotocin (5) 5 to 6 hr after dosing with subsequent lethality of all animals. This finding suggests that the events that occur during the early hyperglycemic phase may be unrelated to the subsequent hypoglycemic and permanent diabetic phases and further emphasizes the importance of the nitroso group in the β-cell cytotoxic action of streptozotocin.

Analog (III) which is referred to as GCNU or NSC 114460, is one of the most promising antitumor agents of the *N*-alkyl nitrosourea family (16) and was previously reported by Schein and coworkers to lack hyperglycemic activity (17). They found that rat blood glucose levels measured 48 hr after the administration of 200 mg/kg of (III) were within the normal range. However, our data with GCNU and its nonacetylated analog (I), known as DCNU or chlorozotocin (18), have shown that transient hyperglycemia can be produced in doses of 100 mg/kg ip in rat glucose tolerance tests. This finding plus the similarity of carbohydrate perturbations suggest that caution should be exercised when these agents are used in the clinical treatment of tumors.

Finally, although the non-nitrosated analogs of streptozotocin, (II) and (IV)-(VIII), have been proven to be inactive against L1210 lymphoid leukemia and against human epidermoid carcinoma of the nasopharynx (cell culture) (19), their pancreatotrophic effect without production of frank diabetes indicates potential utility as pancreatic radiopharmaceuticals. Radioiodinated analogs of several of these streptozotocin derivatives are presently under study with respect to their ability to image islet cell malignancies (20).

Summary. Six non-nitrosated and three nitrosated analogs of the antitumor and diabetogenic agent, streptozotocin, have been found to produce hyperglycemia in fasted rats. The hyperglycemia produced by the non-nitrosated analogs was of early onset and did not result in the production of permanent diabetes. The data emphasizes the important role of the nitroso group of streptozotocin in the production of the hypoglycemic and permanent diabetic phases while the sugar moiety may have a direct influence on production of the early hyperglycemia phase.

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togenicity in Rats of Cell Walls from Several Streptococci Staphylococci and Two Other Bacteria[1] (39360)

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uvant-induced arthritis (AA) in rats :en produced by a single injection of *ıacterium* in water-in-oil emulsion. It wn that *Nocardia* (1) and *Corynebac- rubrum* (2) can replace *Mycobacte-* or AA production. Recently Koga *et* have reported that the cell walls from *ımyces fradiae, Streptomyces lavendu- ıctobacillus plantarum,* and *Staphylo- aureus* were able to produce AA in nd all clinical features were identical se produced by *Mycobacterium*. More ly it has been reported that infection ive *Salmonella enteriditis* was able to :e arthritis in the L-BN rats (4). fore, it should be reasonable to as- :hat a common principle(s) shared by s bacteria and probably located in the ılls, is involved in the pathogenesis of 3ased upon this reasoning we have aken to compare the arthritogenicity ious bacterial cell walls and to deter- vhat components of the cell wall may olved in production of the disease.

following experiments are designed the arthritogenic potentiality of the ılls from seven streptococcal and two lococcal strains and from two other ia.

erials and methods. Animals. Female inbred rats (Microbiological Associ- Bethesda, Md.) were utilized. They :d 150 to 174 g.

wall preparations. The cell walls in the present study were generously :d by Dr. S. Kotani (Dept. of Micro- y, Osaka University Dental School, ı). The preparation of these cell walls is ›ed in detail elsewhere (5).

terial species tested were *Streptococ- :tis (Str. lactis* IFO 12546), *Str. faecalis* (IFO 12580), *Str. mutans* (BHT), *Str. salivarium* (HHT), *Str. salivarius* (IFO 3350), *Str. bovis* (IFO 12058), *Str. thermophilus* (IFO 3535), *Str. pyogens* (group A streptococcus, type 12, S.F. 42 strain), *Staphylococcus aureus (S. aureus* FDA 209P), *S. epidermidis* (ATCC 155), *Bacillus megaterium (B. megaterium* KM), *Micrococcus lysodeikticus (M. lysodeikticus* NTCC 2665), and *Mycobacterium smegmatis (M. smegmatis* ATCC 14468).

Preparation of the hydrosoluble peptidoglycans. Lysozyme-solubilized hydrosoluble products (peptidoglycans) were prepared from *M. smegmatis* and the human mixed strains of *Mycobacterium* (C, DT, PN), according to the method of Adam *et al.* (6) with some modifications (7). The preparation of the hydrosoluble peptidoglycans (PG) from *S. aureus* cell walls is described in detail elsewhere (5). These peptidoglycans were used as skin test antigens in the present study.

Production of AA. Rats were injected into both inguinal lymph nodes with a total volume of 0.01 ml of water-in-oil emulsion, containing 0.1 mg each of cell walls, according to the Newbould technique (8).

The cell walls were triturated well with mortar and pestle and thoroughly mixed in a mineral oil containing 15% of Arlacel A.

A water-in-oil emulsion was thus prepared by the dropwise addition of an equal volume of 0.01 *M* phosphate buffered saline (PBS), pH 7.2, in order to make a final concentration of the cell walls 0.1 mg per 0.01 ml of the water-in-oil emulsion.

Evaluation of AA. The rats were examined daily for 3 to 4 weeks after inoculation to evaluate the onset day of the disease and were graded from 0 to 4 points for each appendage, in accordance with a previous report (9). In general, the arthritogram score below 5 was evaluated as mild and

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[1] s work was supported by USPHS Grant No. 759.

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transient arthritis; 6 to 10, moderate; 11 to 14, severe; and 15 to 20, extremely severe.

Skin testing. Skin tests were performed 21 days after inoculation on a shaved area of the flank by intradermal injection with 0.1 ml of 0.01 *M* PBS, pH 7.2, which contained 50 mg of test antigen. The diameter of the erythema and the induration of the skin test area was carefully recorded at 6 hr for immediate reaction, and 24 and 43 hr for delayed type hypersensitivity, according to the method of Flax *et al.* (10).

Results. Arthritogenicity of various different streptococcal cell walls. As shown in Table I, the cell walls from *Str. bovis*, *Str. lactis*, *Str. mutans*, *Str*, *salivarius* (HHT), *Str. pyogenes*, and *Str. thermophilus* were able to produce mild to severe disease with an incidence of 50, 70, 80, 25 (total for two species), 30, and 70%, respectively. The cell walls from *Str. salivarius* (IFO 3350) and *Str. faecalis* did not produce the disease.

Arthritogenicity of two staphylococcus and miscellaneous bacterial cell walls. As shown in Table II, *M. smegmatis* cell walls and human mixed strains of *Mycobacterium* (C, DT, PN) produced extremely severe disease with 100% of incidence. The clinical signs appeared 6 to 9 days after inoculation and reached a mean high score of 20 and 19.5, respectively.

S. aureus cell walls produced severe disease with an incidence of 100% in one experiment and of 90% in another experiment, while *S. epidermidis* cell walls were unable to produce disease. The cell walls from *B. megaterium* and *M. lysodeikticus* were also found to be nonarthritogenic.

Delayed skin hypersensitivity in rats immunized with various cell walls. The results are shown in Table III. The rats immunized with *M. smegmatis* elicited strong delayed hypersensitivity to peptidoglycans of either *M. smegmatis* or *S. aureus* but not to PPD, while the rats immunized with human mixed strains of *Mycobacterium* (C, DT, PN) elicited strong delayed hypersensitivity to peptidoglycans and PPD. The *S. aureus* cell walls also induced strong delayed hypersensitivity to all peptidoglycans but not to PPD. The cell walls of *S. epidermidis*, *B. megaterium*, and *M. lysodeikticus* did not induce any skin reactions to PPD or peptidoglycans.

Immune response of the rats immunized with various streptococcal cell walls. As can be seen in Table IV, there is a very good correlation between development of the disease and delayed hypersensitivity reactions to the peptidoglycan of human mixed strains of *Mycobacterium* (C, DT, PN). All the rats which developed the disease after inoculation of the cell walls from *Str. thermophilus*, *Str. pyogenes*, and *Str. salivarius* (HHT) elicited a positive delayed hypersensitivity to the peptidoglycan of *Mycobacterium* (C, DT, PN). However, the rats which did not develop the disease after inoculation of the above cell walls showed no skin response except one which was immunized with *Str. salivarius* (HHT). The rats immunized with

TABLE I. ARTHRITIS-INDUCING ABILITY OF VARIOUS STREPTOCOCCAL CELL WALLS.

		Adjuvant Arthritis			
Source of cell wall	Dose[a] (mg/rat)	Incidence	Onset day	(Average)	Mean high score
Str. bovis	0.1	5/10	10-15	(12.0)	14.6
Str. lactis	0.1	7/10	12-14	(13.4)	11.0
Str. mutans	0.1	8/10	12-15	(14.1)	11.5
Str. salivarius[b]	0.1	0/10	–		–
Str. salivarius[c]	0.1	1/10	13	(13.0)	12.0
Str. salivarius[c]	0.1	4/10	12-15	(13.4)	10.5
Str. pyogenes	0.1	3/10	11	(11.0)	12.0
Str. thermophilus	0.1	7/10	12-15	(12.6)	7.9
Str. faecalis	0.1	0/10	–		–
Control[d]		0/10	–		–

[a] Female Lewis rats were immunized in both inguinal lymph nodes with 0.01 ml of a water-in-oil emulsion which contained of 0.1 mg each of bacterial cell walls.

[b] *Str. salivarius* (IFO-3350).

[c] *Str. salivarius* (HHT).

[d] Lewis rats were immunized with a water-in-oil emulsion without any bacteria.

TABLE II. ARTHRITIS-INDUCING ABILITY OF TWO STAPHYLOCOCCAL CELL WALLS AND MISCELLANEOUS BACTERIAL CELL WALLS.

Source of cell wall	Dose[a] (mg/rat)	Adjuvant arthritis: Incidence	Onset day	(Average)	Mean high[b] score
ycobacterium					
M. smegmatis	0.1	10/10	6-8	(7.2)	20.0
C, DT, PN[c]	0.1	10/10	7-9	(7.7)	19.5
phylococcus					
S. aureus	0.1	10/10	9-16	(12.0)	14.4
	0.1	9/10	8-10	(11.7)	13.1
S. epidermidis	0.1	0/10	–		–
	0.1	0/10	–		–
iscellaneous or others					
B. megaterium	0.1	0/10	–		–
M. lysodeikticus	0.1	0/10	–		–

[a] Female Lewis rats were immunized in both inguinal lymph nodes with 0.01 ml of a water-in-oil emulsion ich contains 0.1 mg each of material.

[b] This score was calculated by averaging the highest score of each rat.

[c] Human mixed strains of *Mycobacterium*. The delipidated cells of these Mycobacteria were used instead of cell ll.

TABLE III. IMMUNE RESPONSE OF THE RATS IMMUNIZED WITH VARIOUS BACTERIA.

Immunization[a]	Adjuvant arthritis	48-hr skin reaction[c] Average diameter ± SE (mm): PPD	Hydrosoluble product (peptidoglycan) of: C, DT, PN	*M. smegmatis*	*S. aureus*
. smegmatis	+[d]	Neg.	Neg.	16.0 ± 0.6	10.8 ± 0.5
, DT, PN[b]	+	15.5 ± 1.0	16.0 ± 0.6	12.0 ± 1.2	9.9 ± 0.7[f]
. aureus	+	Neg.	12.8 ± 0.7	15.2 ± 0.9	11.2 ± 0.6
. epidermidis	–[e]	Neg.	Neg.	Neg.	
. lysodeikticus	–	Neg.	Neg.	Neg.	
. megaterium	–	Neg.	Neg.	Neg.	

[a] All rats were immunized in both inguinal lymph nodes with 0.01 ml of water-in-oil emulsion containing 0.1 g each of bacterial cell wall except human mixed strains of Mycobacteria (C, DT, PN).

[b] Delipidated cells were used instead of cell wall.

[c] Skin tests were performed 21 days after inoculation with 0.1 ml of PBS containing 50 μg each of test antigens.

[d] All of the rats developed the disease.

[e] None of the rats developed the disease.

[f] The test antigen utilized here was a different lot of peptidoglycan of *S. aureus* from that used in the other two roups.

ionarthritogenic cell walls such as *Str. saliarius* (KFO 3350) and *Str. faecalis*, and the ontrol normal rats did not elicit any skin eaction. None of the rats in this experiment lemonstrated an immediate type skin reacion.

Discussion. The present data indicate that he cell walls of *Str. thermophilus*, *Str. ovis*, *Str. lactis*, *Str. mutans*, *Str. pyogenes*, nd *Str. salivarius* (HHT) were able to proluce the disease while the cell walls of *Str. aecalis* and *Str. salivarius* (IFO 3350) were nable to do so. The arthritis and other eatures were identical to those produced by *Mycobacterium* (e.g., *M. smegmatis* and human mixed strains of *Mycobacterium*). It was shown in a previous report (3) that the cell walls of *S. aureus* and *Str. pyogenes* produced little or no sign of arthritis. However, we found that cell walls of *S. aureus* did produce severe disease with more than 90% of incidence and *Str. pyogenes* cell walls also produced the disease with over 30% of the animals tested. These discrepancies could be the result of the composition of mineral oil employed. In our present study we used mineral oil containing 15% of Arlacel A, while in the previous study (3) mineral oil containing 5% of Arlacel A was used. In the case of *Mycobacterium* and its

TABLE IV. Immune Response of the Rats Immunized with Various Streptococcal Cell Walls.

Immunization[a]	Adjuvant[b] arthritis	48-Hr skin reaction Average diameter ± SE (mm)		
		PPD	Hydrosoluble product (peptidoglycan) of C, DT, and PN	
Str. thermophilus	+	Neg.	10.6 ± 0.9	(7/7)[c]
	−	Neg.	Neg.	(3/3)[d]
Str. pyogenes	+	Neg.	10.3 ± 0.6	(3/3)[c]
	−	Neg.	Neg.	(7/7)[d]
Str. salivarius (HHT)	+	Neg.	13.5 ± 1.2	(4/4)[c]
	−	Neg.	13.0 ± 0.0	(1/6)[e]
Str. salivarius (IFO-3350)	−	Neg.	Neg.	(10/10)
Str. faecalis	−	Neg.	Neg.	(10/10)
Control[f]	−	Neg.	Neg.	(10/10)

[a] All the rats were immunized in both inguinal lymph nodes with 0.01 ml of water-in-oil emulsion containing 0.1 mg each of bacterial cell wall and 21 days later skin tested with 50 μg each of test antigen.

[b] +, Arthritic rat; −, nonarthritic rat.

[c] Number of rats with positive skin tests/number of arthritic rats.

[d] Number of rats with negative skin tests/number of nonarthritic rats.

[e] Number of rats with positive skin tests/number of nonarthritic rats.

[f] Control rats were immunized with incomplete adjuvant.

wax D, we could not find any difference in their arthritogenicity when the two mineral oil preparations were used. However, the mineral oil containing 15% of Arlacel A seems to give rise to a better water-in-oil emulsion (thick emulsion), at least with the cell walls described above. The higher Arlacel A concentration may be necessary because of the much lower lipid content and a much higher polysaccharide content in these bacterial cell walls as compared to that in the Mycobacterial cell walls (11).

On the other hand, we repeatedly confirmed the observations of Koga *et al.* (3) in which the cell walls from *S. epidermidis*, *B. megaterium*, and *M. lysodeikticus* were nonarthritogenic. The failure of these cell walls to produce the disease may be relevant to the fact that they were unable to replace *Mycobacterium* in complete Freund adjuvant in guinea pigs (5). All of the arthritogenic cell walls used here have been shown to function as adjuvants in guinea pigs both by increasing antibody synthesis and inducing delayed hypersensitivity (cell-mediated immunity) to ovalbumin (5). However, the cell walls of *Str. salivarius* (IFO 3350) and *Str. faecalis* having an adjuvant activity (5) could not produce the disease, as is shown in Table I. Thus the present results provide the view that adjuvanticity is needed for bacterial cell walls to produce the disease, while arthritogenicity requires an additional component which must be a specific antigen responsible for the disease production. One must also consider the possibility that the arthritogenicity of certain bacterial cell walls as described here may be attributed to the amount of this antigen available in the cell walls.

The present results also indicate that a very good correlation exists between development of arthritis and the positive delayed hypersensitivity to the peptidoglycans, with the exception of one rat who was immunized with *Str. salivarius* (HHT) and thereafter did not develope arthritis but exhibited positive skin test (Table III). Thus these results strongly suggest that this delayed hypersensitivity may be involved in the pathogenesis of the disease. Furthermore, it is of interest that the rats immunized with either *S. aureus* cell wall or *M. smegmatis* cell wall elicited strong delayed hypersensitivity to the peptidoglycans from both cell walls. A similar cross-reaction was also observed between the peptidoglycans from the cell walls of *S. aureus* and human mixed strains of Mycobacteria (C, DT, and PN). These cross-reactions among peptidoglycans may be related to the similarity of peptidoglycan structure among bacterial cell walls (11,

he results in Table IV provide an
nal evidence of this cross-reaction.

also clear from the present results
PD and its hypersensitivity *was not*
ed in the pathogenesis of the disease,
e Streptococcal cell walls do not con-
PD-like substances which are specific
cobacterium and did not induce PPD
ensitivity in the rats which developed
disease.

iew of the fact that many bacterial
ons can cause acute infectious arthri-
nan (13), our present study supports
ew that a common structure among
ial cell walls may exert an important
ical role in the development of infec-
rthritis in man. It is also of interest to
that mycoplasma (14) and diphthe-
ganisms (15, 16) can be isolated from
eumatoid arthritic joints in man and
ese organisms can also produce po-
itis in rats (3, 17). Furthermore, it
en shown that there is an increased
ce of septic arthritis in patients with
atoid arthritis (18). Therefore, the
t results, taken together with above
(3, 14–18), provide further support
hypothesis that bacterial infection
e partly involved in the pathogenesis
umatoid arthritis and may have some
ce on the course and development of
ease, possibly in these disorders serv-
her as direct antigenic stimulants or
likely as adjuvants to assist in the
n and/or propagation of another in-
s agent, such as a virus, mycoplasma,
, etc. In this connection, the use of
aracterized cell walls as used in this
del system may also be helpful to
te the pathogenesis of joint disease in
Vork is currently in progress in our
tories along these lines.

mary. Bacterial cell walls from *Str.*
Str. lactis, Str. mutans, Str. thermoph-
r. salivarius, and *Str. pyogenes* were
produce polyarthritis in rats but *Str.*
cell walls were nonarthritogenic. *S.*
cell walls produced extremely severe
. It was also shown that cell walls
from *S. epidermidis, B. megaterium,* and *M. lysodeikticus* were nonarthritogenic. A close correlation was observed between development of arthritis and the delayed hypersensitivity to bacterial peptidoglycans but *not with the PPD* hypersensitivity. It was suggested that the adjuvanticity of bacterial cell walls is needed to induce the disease and that arthritogenicity requires a specific antigen in addition to the presence of an adjuvant-inducing agent.

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Fractionation of Serum Transcobalamins on Charged Cellulose Filters (39361)

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The three transcobalamins (i.e., vitamin B_{12}-binding proteins), TCI, TCII, and TCIII, known to be present in human serum, are difficult to determine by current procedures. The difficulties stem from the unique properties of TCIII, which resembles TCII in its electrophoretic mobility and behavior on DEAE-cellulose batch separation, while immunologically and in its molecular weight it is similar to TCI (1-4). The determination of the three transcobalamins requires therefore a combination of two procedures, a DEAE-cellulose column to separate TCI from TCII and TCIII on one hand, and a sephadex G-200 column to separate TCII from TCIII on the other (1-4). Alternatively, the selective removal of TCII either by precipitation with ammonium sulfate (5, 6) or by adsorption to uncoated charcoal (2) and to silica powder (7), enabled the use of DEAE-cellulose chromatography alone. A DEAE-cellulose column was used to separate all the three binders by changing the eluting buffer several times (8, 9); however, such a method seems too cumbersome for adaptation to the clinical laboratory.

It is already recognized that certain pathological conditions are associated with significant and specific changes in concentrations of the serum transcobalamins. Low unsaturated B_{12}-binding capacity (UBBC) and increase in serum B_{12} concentration bound mostly to TCII is characteristic of hepatocellular damage (10-12). Marked increases in TCI and elevated serum B_{12} are associated with chronic myelogenous leukemia (CML) and acute promyelocytic leukemia (APL) (13-23). Elevated TCIII and normal serum B_{12} values are found in polycythemia vera (PV) and clinical conditions associated with leukocytosis other than leukemia (1, 4, 24).

A rapid and simple procedure for fractionation of the transcobalamins should therefore be of value in the diagnosis of various pathological conditons.

A simple method for the quantitative determination of TCI and TCIII was recently reported by us (25). It is based on the selective adsorption of these transcobalamins to charged filter disks of DEAE-cellulose and cellulose-nitrate, respectively. In the present study the method was modified to determine simultaneously all three B_{12} binders.

Materials and methods. Serum preparation. Sera from normal subjects and from patients suffering from chronic myelogenous leukemia (CML) and polycythemia vera (PV) were rapidly separated and stored at −20°. A large batch of pooled normal serum (100 ml) was incubated for 30 min with excess (5 ng/ml) of nonradioactive vitamin B_{12} and dialyzed overnight against distilled water. This serum will be referred to as B_{12}-saturated serum and was used as an additional coating procedure for the charcoal (see below) to prevent adsorption of TCII from incubation mixtures containing small amounts (0.01 ml) of serum.

^{57}Co B_{12}. ^{57}Co B_{12} of high specific activity between 135-200 $\mu Ci/\mu g$ (The Radiochemical Centre, Amersham, Bucks, England) was used. Batches of 10 μCi were diluted with water to a final concentration of 10,000 pg B_{12}/ml and stored in the refrigerator when not in use.

Determination of UBBC and transcobalamin distribution by the filter stack. The filter stack is an assortment of wet charged cellulose disks of 25 mm in diameter (25) placed on a millipore type filter holder apparatus (The Tamar Co., Jerusalem, Israel). The stack is composed of a single cellulose-nitrate filter (Schleicher and Schüll, Dassel, Germany) which is placed on top of three DE-81 filters (The Whatman Biochemicals Ltd., Maidstone, Kent, England) rather than two as used previously (25), as it enables a complete recovery of TCI and TCIII

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ons. Buffer solutions for the filter
s (see below) were prepared using glass
ed water and filtered through a cellu-
itrate filter to remove particles which
interfere with the assay.
plicate samples of the serum (0.01 ml
were incubated for 30 min at 37° with
s ^{57}Co B_{12} (50–100 pg/0.01 ml), 0.2 ml
l *M* sodium borate buffer (pH 8.5) and
to a final volume of 0.4 ml; alterna-
, larger aliquots of sera and radio B_{12}
d 1:10 or so may be taken. After incu-
n, each reaction mixture was diluted
10–12 ml of the borate buffer and
d, by applying vacuum, through the
stack described above; a 1/3 HP air
m pump and a trap for collecting the
s of unbound ^{57}Co B_{12} was used.
s of unbound radio-vitamin were re-
d by washing the filter stack twice,
10 ml of the same buffer. One washing
10 ml of buffer was usually sufficient to
ve all unbound radioactivity as assayed
tly in the filtrate; however, to assure
ant and complete removal of the un-
d ^{57}Co B_{12} two additional washings are
mended. The unsaturated B_{12}-binding
ity (UBBC), expressed as amount of
rams of ^{57}Co B_{12} bound per milliliter of
, was calculated from the radioactivity
ed by the stack. The percentage distri-
n of the binders was determined from
adioactivities retained by each filter-
efore and after subjecting the stack to
r washing with acid potassium phos-
buffer as detailed in the text.

ermination of UBBC by the coated-
al adsorption method. The reaction
re and incubation conditions were as
ibed above. After the incubation pe-
0.5 ml of the "B_{12}-saturated serum"
oned above was added to the reaction
re. Excess of unbound ^{57}Co B_{12} was
removed by adding of 0.5-ml suspen-
f Dextran 80-coated charcoal (1 g of
an 80/5 g of Norit A/100 ml of salt
on) (26). After centrifugation the
C was determined by measuring the
ctivity of the supernatant.

ctive removal of TCII and estimation
I and TCIII by chromatography.
obalamin II was selectively removed
he sera by treatment with Quso G32 (a microfine precipitated silica which was generously supplied by Dr. Victor Herbert), by adding 20 mg of the silica per 1 ml of serum and shaking the suspension for 30 min (7). After centrifugation, the supernatant was used to determine either the total concentration of TCI and TCIII or their individual components after separation on a DE-52 column or by specific elution from the DE-81 filter stacks as described in the Results.

The separation of TCII from TCI and TCIII was also carried out using the Sephadex G-200 column chromatography described by Hom *et al.* (19).

Results. Adsorption of ^{57}Co B_{12}-transcobalamin complexes to charged cellulose filters. A prerequisite for determination of the three transcobalamins is that they adsorb both quantitatively and selectively to the filter stack. The UBBC values of various sera containing quantitatively different assortments of transcobalamins were therefore determined by the filter stack procedure and the results compared with those obtained by the coated-charcoal adsorption method. The good correlation obtained (Table I) indicates that the three binders are quantitatively adsorbed to the filters.

The selective retention of TCII by the cellulose-nitrate filters is shown in Tables I and II. Removal of TCII with Quso G32 resulted in reduction of 90% or more of the radioactivity retained by the cellulose-nitrate filter (Table I). Furthermore, the radioactivities of fractions from Sephadex G-200 column containing TCII only were retained exclusively by the cellulose-nitrate filters (Table II). On the other hand, Quso G32 treatment did not reduce significantly the radioactivities adsorbed to the DE-81 filters, except for the serum from one CML patient (C.G.), to be dealt with separately (Table I). Also the radioactivities of the Sephadex G-200 fractions containing both TCI and TCIII were adsorbed quantitatively to the DE-81 filters (Table II).

The capacities of each filter-type to retain the respective transcobalamins from increasing amounts of sera is shown in Fig. 1. As seen, a quantitative and selective adsorption was obtained with up to 0.015 ml of serum; when higher amounts of serum were used the retention was not as quantitative due to the exclusion of the B_{12} binders from the filters by the serum proteins.

Selective desorption of TCIII from the DE-81 filters. The DE-81 filters to which both TCI and TCIII adsorb are chemically similar to the granular DE-52, which is used for the separation of the two. However, since the retention of TCIII by DEAE-cellulose is weaker than that of TCI, conditions were explored for the selective desorption of TCIII from the DEAE filters. Several filter stacks to which the transcobalamins from sera of patients with CML and PV were adsorbed, were subjected to further washing with phosphate solutions of varying pH and molarity.

While TCII was strongly retained by the cellulose-nitrate filter both TCI and TCIII were desorbed to a different degree from the DE-81 filters. The data (Figs. 2 and 3)

TABLE I. UNSATURATED B_{12} BINDING CAPACITY (UBBC) OF VARIOUS SERA AS DETERMINED BY THE FILTER STACK AND THE CHARCOAL ADSORPTION METHOD.[a]

		UBBC (pg/ml serum)			
		Filter stack			
Subject	Quso G32 treatment	Cellulose-nitrate (CN)	DE-81	Total (CN + DE-81)	Charcoal
H.I. (normal)	−	1750	110	1860	1800
	+	150	110	260	300
S.I. (normal)	−	715	1100	1815	1900
	+	85	900	985	900
C.G. (CML)	−	850	1800	2650	2800
	+	90	1400	1490	1490
R.P. (CML)	−	370	5500	5870	5700
	+	0	5600	5600	5650
E.R. (PV)	−	800	3500	4300	4500
	+	70	3560	3630	3540
G.I. (PV)	−	770	3900	4760	4800
	+	35	3800	3835	3750

[a] Sera (0.02 ml) were incubated for 30 min at 37° with ^{57}Co B_{12} (150 pg) and 0.1 M sodium borate buffer (pH 8.5) in a total volume of 0.4 ml. Half of the incubation mixture was tested for UBBC by removing unbound radioactivity with the coated charcoal (See Materials and Methods). The other half was assayed by the filter stack procedure as described. Additional samples of these sera were treated with Quso G32 prior to incubation and UBBC determination.

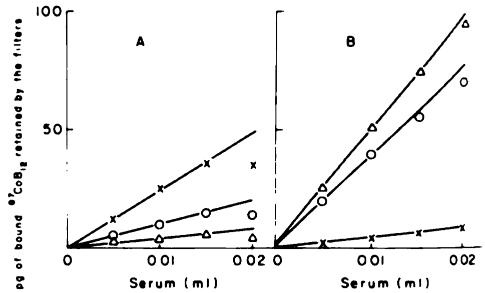

FIG. 1. Retention of transcobalamins by the filters of cellulose-nitrate and DE-81 as a function of serum concentration. Samples of sera outlined below (0.08 ml) were incubated each with 800 pg of ^{57}Co B_{12}. After adjusting the volume to 8 ml with 0.1 M sodium borate buffer (pH 8.5), aliquots from 0.25 to 2 ml were diluted with the borate buffer and passed through the filter stacks as outlined in Materials and Methods. (A) ^{57}Co B_{12} retained by the cellulose-nitrate filter; (B) ^{57}Co B_{12} retained by the DE-81 filters. The following sera were used: A.I. (normal), x; C.G. (CML), △; and E.R. (PV), O.

TABLE II. ADSORPTION OF ^{57}Co B_{12}-TRANSCOBALAMIN COMPLEXES TO CELLULOSE-NITRATE AND DE-81 FILTERS.[a]

		Radioactivity (cpm)		
			Retained	
Subject	Transcobalamin types	Added	Cellulose-nitrate	DE-81
R.G. (Normal)	TCII	9000	8550	100
R.P. (CML)	TCII	8700	8600	0
W.B. (PV)	TCII	5600	5600	0
R.G. (Normal)	TCI + TCIII	2000	0	2000
R.P. (CML)	TCI (mainly)[b] + TCIII	9300	0	9200
W.B. (PV)	TCIII (mainly)[c] + TCI	5900	0	5900

[a] The sera (1 ml) were incubated with a subsaturating concentration of ^{57}Co B_{12} (300 pg/ml) and fractionated on columns of Sephadex G-200 by the procedure of Hom *et al.* (19). Pooled fractions from regions of high-(TCI and TCIII) and low-(TCII) molecular weight were dialyzed; the contents of bags were concentrated by aeration to about 2 ml, then redialyzed against distilled water. Aliquots of 0.1 or 0.2 ml, depending on the radioactivity of the fractions, were passed through filter stacks at pH 8.5, and the radioactivity of each filter type was determined.

[b] 90% and [c]80% as determined by DE-52 column chromatography.

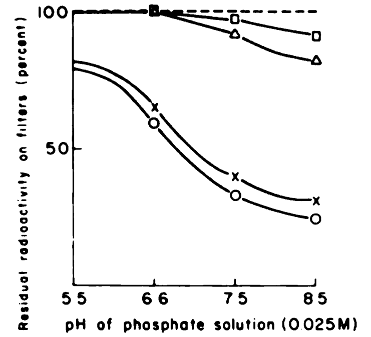

FIG. 2. Effect of pH of the phosphate solution on elution of transcobalamins from the filter stack. Samples of sera (0.1 ml) were incubated with 1000 pg of ^{57}Co B_{12} in presence of borate buffer. Final volume was 2.0 ml. Aliquots of 0.2 ml were diluted with the borate buffer (pH 8.5) and passed through filter stacks as described. One stack, which served as control, was washed further with 15 ml of the same buffer solution. The others were washed each with a 15-ml solution of a different pH value (varying from 5.5 to 8.5) of 0.025 *M* potassium phosphate. The residual radioactivity of each filter type was compared to that of the control (100% retention). The dashed line in the figure represents the residual radioactivity on the cellulose-nitrate filters of all sera tested. Solid lines represent residual radioactivities on the DE-81 filters of the following sera: W.B. (PV), ○; G.L. (PV), x; R.P. (CML), □; and C.D. (CML) △.

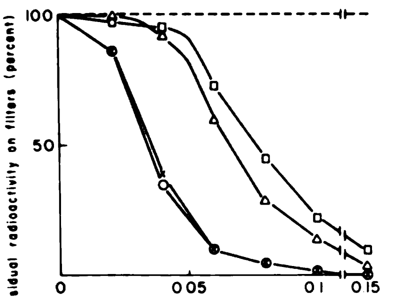

FIG. 3. Effect of phosphate (KH_2PO_4) concentration on elution of transcobalamins from the filters. The experimental procedure and symbols used were as described in the Legend to Fig. 2, except that increasing concentrations of KH_2PO_4 (pH 4.6) were used as eluant.

show that the retention of the binder which predominates in the PV sera (TCIII) was much weaker than that of TCI, the main B_{12} binder in CML. Employing a pH, at which the degree of ionization of the phosphates is minimal (i.e., KH_2PO_4, pH 4.6), the effect of increase in the concentration of the wash solution on the retention of the B_{12} binders was tested (Fig. 3). The data in this figure suggested that TCIII was selectively desorbed from the filters by a 0.05 *M* KH_2PO_4 solution; at this concentration most of the binder from the PV serum was removed, yet that of the CML serum was mostly retained. Confirmation of the above is shown in Table III. Sera from normal subjects and from patients with CML and PV were treated with Quso G32 and its TCI and TCIII components were determined after DE-52 column chromatography. The values obtained were either similar to or identical with those determined by the filter stack using 0.05 *M* KH_2PO_4 solution for desorption of TCIII.

The UBBC values and the relative distribution of the three B_{12} binders in normal, CML, and PV sera as determined by the filter stack are listed in Table IV. As seen, these sera are clearly distinguishable when assayed by the described filter stack procedure.

TABLE III. DISTRIBUTION OF TCI AND TCIII AFTER REMOVAL OF TCII BY QUSO G32.[a]

	Distribution of transcobalamins (percentage)			
	DE-52 column		Filter stack	
Subject	TCI	TCIII	Retained	Desorbed
B.R. (normal)	23	77	22	78
B.B. (normal)	46	54	42	58
Z.M. (normal)	43	57	44	56
R.P. (CML)	95	5	91	9
E.X. (CML)	91	9	90	10
C.D. (CML)	88	12	81	19
W.B. (PV)	19	81	22	78
J.J. (PV)	25	75	24	76
G.L. (PV)	21	79	26	74

[a] All sera (1 ml each) were treated with Quso G32; 0.5-ml aliquots were incubated with 150 pg of ^{57}Co B_{12} and the TCI and TCIII distribution was determined after DE-52 column chromatography. Duplicate samples of the same sera (0.01 ml each) were also incubated with ^{57}Co B_{12} (100 pg/0.01 ml), then passed through the filter stack, and washed with borate buffer. One stack from each duplicate was further washed with 15 ml of 0.05 *M* KH_2PO_4 (pH 4.6). There was no detectable radioactivity on the cellulose-nitrate filters. The percentage of transcobalamin retained by the DE-81 filters was calculated from the radioactivities obtained on these filters prior and after the elution with the acid phosphate solution.

TABLE IV. UBBC AND PERCENTAGE DISTRIBUTION OF THE THREE TRANSCOBALAMINS OF VARIOUS SERA AS DETERMINED BY THE FILTER STACK.[a]

Subject	UBBC (pg B_{12}/ml serum)	TCII	TCI (percentage)	TCIII
B.R. (normal)	2300	70	7	23
B.B. (normal)	1760	81	8	11
Z.M. (normal)	1900	75	11	14
H.Z. (CML)	4430	17	80	3
E.X. (CML)	7000	30	66	4
C.D. (CML)	6660	24	65	11
E.N. (PV)	8600	14	19	67
W.B. (PV)	7000	12	14	74
H.S. (PV)	6300	14	10	76

[a] The experimental procedures were as described in Table III except that treatment with Quso G32 was omitted.

Discussion. The fractionation of the three transcobalamins found in human serum by the described filter stack can be regarded as a combination of several procedures which were scaled down for use of small amounts of sera.

The adsorption of TCII to the cellulose-nitrate filter is due probably to nonelectrostatic attraction of the binder by the filter. The pH (8.5) of the filtration solution is too high for TCII (p*I*, 5.4–6.6) (27) to be attracted by electrostatic forces to the negatively charged filter. Consistent with the above was the failure of potassium phosphate solutions of varying pH to elute TCII from the filter (Figs. 2 and 3).

The treatment with solid adsorbing agents such as Quso G32 yielded similar results, although the mechanism of adsorption of TCII is probably not identical. In some cases, of which one is given in Table I (C.G.), the treatment with Quso G32 resulted in the adsorption of more transcobalamin than observed with the cellulose-nitrate filter. Further information, not brought here, indicates that the extra binding adsorbed to the Quso G32 was definitely not due to TCII and it represents rather the TCI-TCIII group.

Contrary to the strong adsorption of TCII to the cellulose-nitrate filter, that of TCI and TCIII to the DEAE-cellulose filters is due to electrostatic attraction. As in the case of DEAE-cellulose column chromatography specific desorption of TCIII is achieved because of the weaker retention of the latter (p*I*, 3.4–4.0) (6, 27) by the DEAE filters; thus, a solution of moderate ionic strength, such as 0.05 *M* KH_2PO_4 enables TCIII desorption without eluting TCI (p*I*, 2.9–3.1) (6, 27).

While TCII is a clearly defined and distinct protein entity (6), TCI and TCIII may be fractions of the same protein, varying in their isoelectric mobilities (27) and perhaps in additional properties. Stenman (27) suggests that the latter transcobalamins be named cobalophilins rather than R binders, as no definite transport function can as yet be ascribed to them and they do not necessarily show rapid electrophoretic mobility.

The filter stack method, in spite of its simplicity, gave similar results when compared with the more laborious procedures such as the combination of Quso G32 and DEAE-cellulose chromatography. DEAE chromatography alone was inferior in our hands as TCII was partly retained by the column and was eluted together with TCI. The method described is not only simple, but also rapid, and over 100 samples can be tested daily. The quantitative distribution of the B_{12} binders in sera from various pathological conditions is now being investigated on a larger scale. Also effects of different drugs currently used in the therapy of various myeloproliferative diseases on the distribution of the transcobalamins will be studied.

Summary. A simple and rapid fractionation procedure of the three transcobalamins, TCI, TCII, and TCIII, of human serum was achieved by filtration through a stack of charged cellulose filters composed of one cellulose-nitrate and three DEAE-cellulose (DE-81) disks.

A reaction mixture containing microliter amounts of serum was incubated with excess of ^{57}Co B_{12} of high specific activity, diluted with 0.1 *M* sodium borate buffer (pH 8.5), and passed through the filter stack by applying vacuum. Under these conditions TCII is selectively and quantitatively adsorbed to the cellulose-nitrate filter while both TCI and TCIII adsorb to the DE-81 filters. In the second step TCIII is selectively de-

sorbed from the latter filters by a 0.05 *M* monopotassium phosphate solution of pH 4.6.

Using sera of different distribution of transcobalamins the data obtained were comparable to those determined by the more laborious methods employing DE-52 column chromatography combined with procedures to remove TCII.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 210-212 (1976)

Plasma Steroid Concentrations in Conscious and Anesthetized Rabbits[1] (39362)

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Many experiments today involve the collection of blood samples from anesthetized animals. Sometimes when these data are compared to those obtained from conscious animals, discrepancies are apparent. It is very possible that some artifacts may be introduced by anesthetics because they are general depressants. For this reason we designed this experiment to examine if there were any significant differences in plasma estrogen (E), androgens (A), and progestin (P) concentrations with time in conscious and anesthetized animals. Furthermore, we compared the steroid responses between animals injected with buffer and LH because the action of gonadotrophins, the release of steroids, and the action of anesthetics all occur at the membrane level (1, 2). Animals were anesthetized with either halothane or sodium pentobarbital (Nembutal). These anesthetics were chosen because they are commonly used anesthetics and have different modes of action (3).

Materials and methods. Adult female rabbits, weighing 3.5 to 5.0 kg, which had free access to water and feed, were used. At least 21 days before use, rabbits were isolated in individual cages. To facilitate the sampling of blood, silastic cannulas were introduced into the right jugular under nembutal anesthesia and externalized dorsally between the ears. Six to nine animals were used for each treatment group. Rabbits were randomly assigned to one of three groups, conscious, nembutal- or halothane-anesthetized, and bled two days after the insertion of cannulas. Anesthesia was induced by either injecting (iv) approximately 2.5 cc of nembutal (Holmes Laboratories) or by inhalation of halothane (Ayerst) in a close circuit (Foregger). Initially for several minutes halothane and oxygen were presented at a rate of 200 cc/*M*% and L/M but were then reduced to 5 cc/*M*% and 0.250 L/M respectively. The level of anesthesia was identical to that used for general surgery. Blood was removed at a constant rate of 0.5 ml per minute by means of an infusion pump. Animals were bled for 15-min intervals before and at 10 and 45 min after the injection of buffer or LH (10 μg/kg) (NIH-S18). After the blood was centrifuged, plasma was removed, frozen, and later assayed for E, A, and P. Statistical analysis was done by analysis of variance.

Results. In Fig. 1 are shown the E, A, and P plasma concentrations before and after the injection of buffer into rabbits that were either conscious, or anesthetized with nembutal or halothane. The E concentrations showed no time or treatment effect. On the other hand, there was some treatment effect observed for A. While there were no significant differences between conscious and halothane-anesthetized animals, plasma levels were significantly different ($P < 0.01$) between the nembutal and halothane-anesthetized animals. However, the greatest treatment effect was noted in the plasma P concentrations. Here the P levels in halothane-anesthetized animals were significantly ($P < 0.01$) elevated over those for the conscious and nembutal-anesthetized rabbits.

In the second experiment we were curious to see if the conscious or anesthetized condition affected the steroid levels following an injection of LH (10 μg/kg). As shown in Fig. 2, no differences were observed in E concentration among the various treatments either before or after the injection of LH. In contrast, significant increases ($P < 0.01$) in A and P occurred after the injection of LH. Though there was a further increase in A and P with time (45-60-min sampling period), the increase was not significantly different from the previous sampling period (10-25-min). A and P concentrations in the conscious and anesthetized animals after LH were not different. In other words, the

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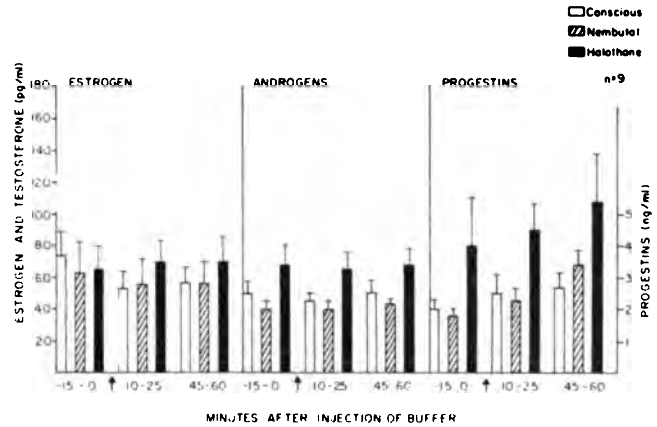

FIG. 1. Peripheral steroid concentrations in conscious and nembutal- and halothane-anesthetized animals before and after the injection of buffer. *n* Indicates the number of animals used in each group. Vertical bars indicate standard errors. The arrow indicates the time of injection of buffer.

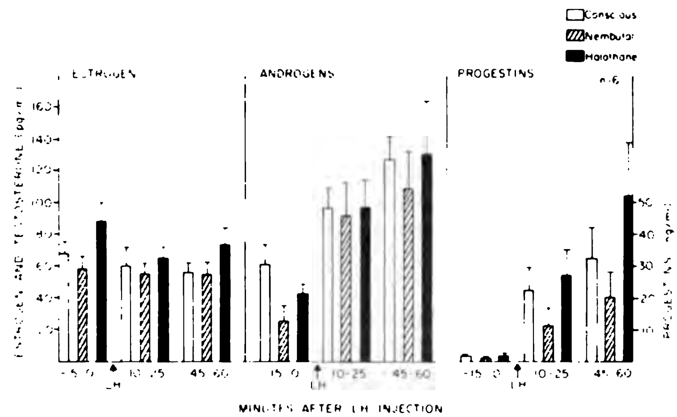

FIG. 2. Peripheral steroid concentrations in conscious and nembutal- and halothane-anesthetized animals before and after the injection of LH. *n* Indicates the number of animals used in each group. Vertical bars indicate standard errors. The arrow indicates the time of injection of LH.

condition of the animal did not affect the steroid response to gonadotrophin stimulation.

Discussion. In these experiments we measured plasma E, A, and P concentration in rabbits that were either conscious or anesthetized with nembutal or halothane. We also compared steroid responses in intact rabbits injected with either buffer or LH. Of the three steroids measured plasma E levels were not altered either by the state of the animal (conscious or anesthetized) or following gonadotrophin stimulation. Since changes in E levels following a systemic injection of LH as measured in the ovarian venous blood increase only twofold (unpublished data), it is not surprising that there is no change in peripheral plasma E concentrations following LH. Similarly, any changes caused by anesthesia may be so minute as not to be observed in the peripheral plasma. On the other hand, A levels were slightly, but not significantly, elevated under halothane anesthesia in Experiment 1. For some unexplainable reason this response was not seen in the first group of samples taken in the second experiment (see Fig. 2) before the injection of LH. This variation in A levels may reflect the variation among the animals or may result from the pooling of the data. The slightly elevated androgen levels observed in halothane-anesthetized animals in Experiment 1 do agree with the results, but not the interpretation, of Free and Tillson (4). They measured secretion rates of testosterone in conscious and halothane-anesthetized rats and observed slightly lower testosterone levels in the conscious rats which they attributed to stress in the conscious rats. On the other hand, while we did not observe any highly significant changes in A levels, Oyama (5) and Oyama *et al.* (6), reported that testosterone concentrations in human plasma decreased 12% from control values during 45 min of halothane anesthesia and continued to fall to 80% of the preinduction levels. These low values continued to fall and persisted for 1 week postoperatively. It is difficult, if not impossible, to draw any comparison among these experiments because of differences in species and anesthetics used, sampling procedures, levels of surgical stress, and duration of the anesthesia.

P concentrations in the halothane-anesthetized rabbits were significantly elevated over those measured in conscious and nembutal-anesthetized rabbits in Experiment 1. In Experiment 2, in which rabbits were injected with LH, this treatment effect, though most likely present, was overridden by the tremendous increase in P levels following gonadotrophin stimulation. These significant differences in P levels in Experiment 1 between the nembutal and halothane anesthetized rabbits may reflect the different physiological response to the two anesthetics. While nembutal is a barbiturate and general depressant, halothane, a voltatile anesthetic, induces delirium unless sedation is first induced by a barbiturate.

In conclusion, we found that P levels were elevated in animals anesthetized with halothane. This highly significant increase in P, but not in E and A, may be the result of stress. We are now investigating whether the ovary or the adrenal is the principal source of the elevated P levels in halothane-anesthetized animals. However, one must be cautious in extrapolating these results to other species because of species differences and the lack of sufficient data regarding hormonal responses following induction of anesthesia.

Summary. The ovarian steroids, estrogen, androgen, and progestin, were measured in the peripheral plasma of adult female rabbits that were either conscious or anesthetized with sodium pentobarbital (nembutal) or halothane. Concentrations of estrogen, androgens, and progestin were determined before and at 10 and 45 min after systemic injection of either buffer or LH. In the controls androgen levels were significantly different between animals anesthetized with nembutal and halothane. However, the greatest treatment effect was noted in plasma progestin concentrations which were significantly elevated in halothane-anesthetized animals in comparison to the conscious and nembutal-anesthetized animals. Following LH, the androgen and progestin levels were significantly elevated over basal levels. Most likely the treatment effect observed in the controls was still present but was overridden by the increased release of steroids following gonadotrophin stimulation. This study suggests that halothane, in contrast to nembutal, does significantly elevate peripheral progestin and androgen levels.

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S OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 213-217 (1976)

# of Intravenous C-Terminal Octapeptide of Cholecystokinin and Intraduodenal Ricinoleic Acid on Contractile Activity of the Dog Intestine (39363)

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tractions of the circular smooth mus- the gastrointestinal (GI) tract are re- substantially in the unanesthetized ter oral cathartic doses of castor oil order to study further the source of ooth muscle inhibition, we conducted s of experiments in which the active ient of castor oil, ricinoleic acid (12- ;yoleic acid), was infused directly into odenum of dogs previously fed a meat (2). After a brief (<2 min), initial ation, the fatty acid produced a pro- (20 min) inhibition of food-induced ctile activity over a considerable por- the GI tract. Ricinoleic acid, there- fused into the lumen of the proximal intestine altered muscle contractions s of the GI tract removed from the n site. This suggested that the effects tor oil and ricinoleic acid on GI muscle *in vivo* were mediated indi- possibly by a duodenal hormone.

study was conducted to compare the s in ongoing digestive contractile ac- after intraduodenal (ID) administra- ricinoleic acid and intravenous injec- the C-terminal octapeptide of chole- inin (CCK-OP). We have found that rocedures produce similar changes in gestive contractile patterns of the intestine and that the initial stimula- sponse after both procedures is medi- rough a cholinergic mechanism.

rials and methods. Extraluminal gage transducers were implanted in ale, mixed breed dogs weighing 10 2 kg. The procedure was performed aseptic surgical conditions with the s anesthetized with sodium pentobar- 30 mg/kg iv). A detailed account of ucer construction and implantation has been published (3). The units were sewn onto the serosal surface of the organ to be studied along the transverse axis to monitor the contractile activity of the circular smooth muscle. Eight transducers were implanted in each animal and positioned on the proximal jejunum (6 and 12 cm caudad to the ligament of Treitz), on the middle jejunum (60 and 66 cm caudad to the ligament of Treitz), on the terminal ileum (14 and 20 cm orad to the ileocecal valve), and on the proximal colon (6 and 12 cm caudad to the ileocecal valve). In addition, each animal was implanted with a duodenal cannula placed approximately 8 cm distal to the gastroduodenal junction. The contractile activity of the circular smooth muscle was recorded by a Beckman Dynograph (Type R411).

The animals were housed in a constant temperature environment and were maintained on a 12-hr light-dark cycle. They had access to water *ad libitum* and received a daily ration of dry dog food. Experiments were performed on the unanesthetized animals no sooner than 10 days after surgery. Experiments were begun between 1 and 3 PM, and the animals lay quietly in a limited access laboratory for the duration of each experiment.

One-half hour before each experiment the animal was fed 200 g of canned dog food to induce continuous contractile activity in the areas monitored. Alterations in digestive contractile patterns were studied after the following procedures: 1) iv bolus injection of CCK-OP, 50 or 500 ng/kg; 2) repeated iv bolus injections of CCK-OP (50 ng/kg) spaced at 5-min intervals; 3) multiple ID bolus infusions of sodium ricinoleate (500 mg, Pfaltz-Bauer, Inc.) at 5-min intervals; 4) the action of atropine sulfate (100 μg/kg sc) on the motor effects produced by iv CCK-OP or ID ricinoleic acid; 5) the action of hexamethonium (10 mg/kg sc) on the motor effects of ID ricinoleic acid. For

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procedure 3, the sodium salt of ricinoleic acid was dissolved in 30 ml of isotonic saline and infused into the duodenal cannula over a 2-min period. A 30-ml volume of isotonic saline adjusted to the pH of the fatty acid solution (9.5) served as control. Each procedure was repeated at least twice in each animal.

Results. CCK-OP produced a biphasic response when given as an iv bolus (Fig. 1). At 50 ng/kg, there was an initial stimulation of digestive contractile activity in the proximal and middle jejunum followed by a post-stimulatory inhibition (PSI). The digestive patterns of the terminal ileum and proximal colon were unaffected. A tenfold increase in dose (500 ng/kg) produced a greater initial stimulation followed by a longer PSI. In contrast to the lower dose, the higher dose affected all areas (Fig. 1). The higher dose also caused the animals to lose consciousness momentarily, apparently due to a fall in blood pressure.

Repeated iv bolus injections of CCK-OP always induced the characteristic response (Fig. 2, upper tracings) of initial stimulation followed by post-stimulatory inhibition. ID bolus infusion of ricinoleic acid affected digestive activity in exactly the same manner. However, repeated ID infusions of the fatty acid produced increasingly diminished stimulatory responses (Fig. 2, bottom tracings). The reduced responses following the second and third ID infusion of ricinoleic acid occurred after an increased time period. ID infusion of control saline did not affect digestive contractile activity.

Atropine completely antagonized the motor effects of iv CCK-OP and ID ricinoleic acid (Fig. 3, upper tracings); hexamethonium only partially antagonized the effects of ID ricinoleic acid (Fig. 3, bottom tracings).

Discussion. The gastrointestinal hormone, cholecystokinin, is released into the peripheral circulation by the presence of fatty acids or amino acids in the lumen of the small intestine (4, 5). Both the hormone and its C-terminal octapeptide have been found to stimulate the contractile activity of the small intestine and colonic smooth muscle (6–9). These effects have been confirmed in the present study. However, we make three additional observations regarding the motor actions of CCK-OP administered by rapid iv bolus: 1) the proximal and

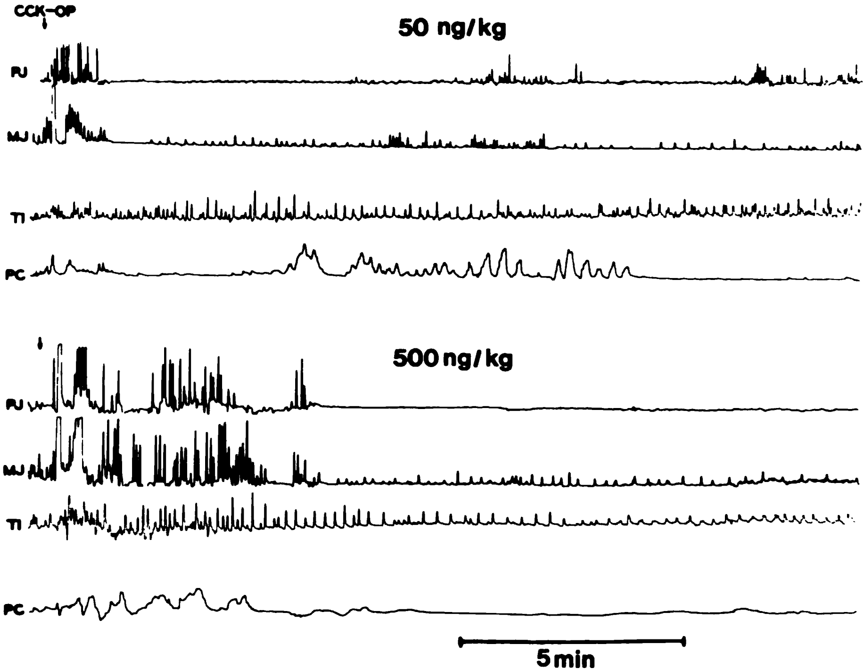

FIG. 1. The effects of iv bolus injection of CCK-OP (50 and 500 ng/kg) on the digestive contractile activity of the proximal (PJ) and middle jejunum (MJ), terminal ileum (TI), and proximal colon (PC).

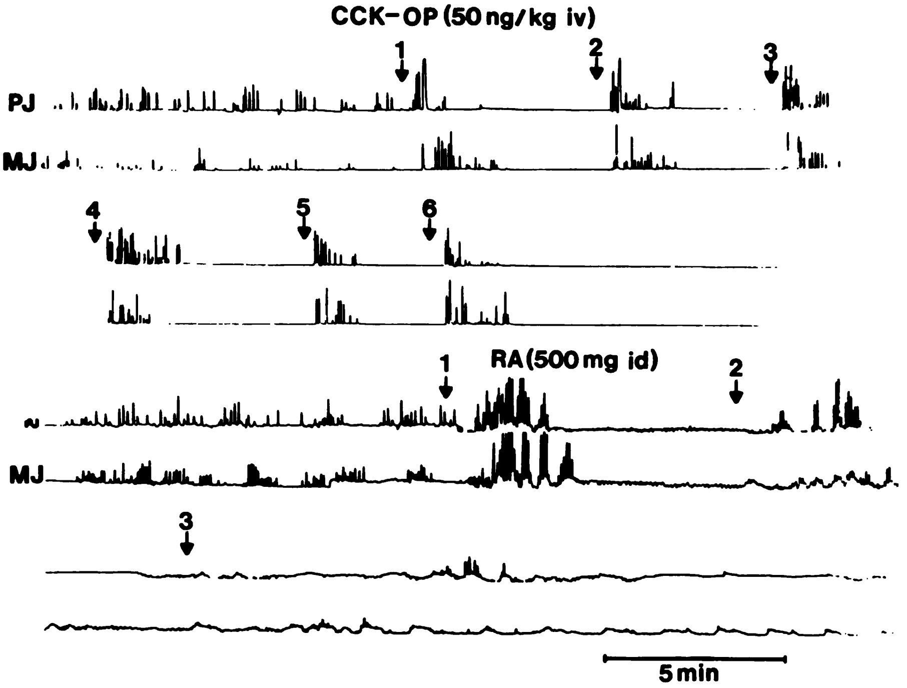

FIG. 2. The top tracings show the effects of six successive iv bolus injections of CCK-OP on the digestive contractile activity of the proximal (PJ) and middle jejunum (MJ). The bottom tracings show the response after the first and the diminished stimulatory responses following the second and third intraduodenal (id) bolus infusions (2-min duration) of ricinoleic acid (RA). Note the greater time period required for the responses to the second and third intraduodenal bolus of RA.

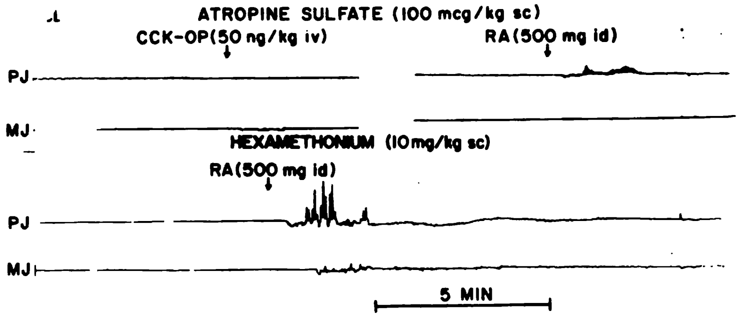

FIG. 3. The action of atropine sulfate on the effects of iv CCK-OP and intraduodenal ricinoleic acid (RA) on the digestive contractile activity of the proximal (PJ) and middle jejunum (MJ) is shown in the top tracings. The bottom tracings demonstrate the inability of hexamethonium to antagonize the intraduodenal ricinoleic acid response completely.

middle small intestine are affected preferentially by lower doses of the compound; 2) when the effects of iv CCK-OP are superimposed upon an established, physiologic level of contractile activity induced by food, the initial stimulation of motor activity is followed by inhibition; 3) the motor actions of iv CCK-OP showed marked similarities to the motor responses evoked by infusion of ricinoleic acid directly into the duodenum. The latter observation suggests that the motor effects following infusion of a fatty acid into the proximal small intestine may result predominately from release of endogenous cholecystokinin.

The motor response after ID ricinoleic

acid was similar in all but one respect to the motor effects of iv CCK-OP. Unlike repeated iv administrations of the octapeptide, successive challenges with ID fatty acid failed to produce continued responses. If the stimulatory motor actions of ID ricinoleic acid do result from the release of endogenous cholecystokinin, then feeding a meat meal and the subsequent presence of the contents in the small intestine must not release all stores of the hormone completely. It may require repeated infusion of a potent releaser of cholecystokinin, such as a fatty acid, directly into the small intestine to exhaust this residual store of hormone. The greater time period required for the diminished responses to the second and third ID bolus of fatty acid might result from the release of more distal stores of cholecystokinin. This is compatible with the observation that releasable cholecystokinin extends beyond the most proximal small intestine and decreases with increasing distance from the pylorus (10). Alternately, the failure of continued ID infusions of ricinoleic acid to produce a response might result from a desensitization of the hormone release mechanism.

The stimulant effect of cholecystokinin on GI smooth muscle appears to be cholinergically mediated. Recent investigations present evidence that CCK interacts with non-nicotinic receptors on the ganglion cells of Auerbach's plexus to release acetylcholine at neuroeffector junctions (11-13). The present investigation supports these findings since atropine antagonized the stimulatory response to iv CCK-OP. Furthermore, atropine, but not hexamethonium antagonized the motor effects of ID ricinoleic acid. Thus the stimulatory effects of both ID ricinoleic acid and iv CCK-OP are mediated by a cholinergic mechanism.

The source of the PSI following iv CCK-OP or ID ricinoleic acid noted in this study is unknown. The fact that the response was obtainable after both procedures suggests that its source is a high circulating level of cholecystokinin. However, the PSI response may not be a direct action of the hormone. Glucagon, a hormone known to inhibit motor activity of the small intestine (14, 15) is released into the circulation during high circulating levels of cholecystokinin (16, 17) and may be the ultimate source of the response.

Equal quantities of oleic acid and ricinoleic acid infused into the proximal small intestine produce similar alterations in digestive contractile patterns. Quantitatively, however, the inhibitory phase of the response to ricinoleic acid is much greater (2). Similar differences are noted after oral administration of the fatty acids. Oral oleic acid (10 g) alters digestive contractile patterns briefly. Oral ricinoleic acid (10 g) produces a prolonged (4 to 6 hr) inhibition of digestive contractile patterns after which the animal produces a wet stool (2). These observations indicate that the major difference in the motor patterns induced by a diarrheogenic and nondiarrheogenic fatty acid, either orally or intraduodenally administered, is the greater ability of the diarrheogenic fatty acid to maintain the period of circular smooth muscle inhibition. Whether this difference in turn reflects differences in hormone releasing ability between the fatty acids remains to be determined.

Summary. Intraduodenal infusion of ricinoleic acid and iv bolus injection of the C-terminal octapeptide of cholecystokinin produce markedly similar alterations in the digestive contractile patterns of the GI tract of the unanesthetized dog. The brief, initial stimulation of contractile activity in the proximal small intestine following both procedures is mediated through a cholinergic mechanism. The stimulatory response is followed by an inhibition of digestive contractile activity of unknown origin. These observations suggest the possibility that the GI hormone, cholecystokinin, may mediate the intestinal motor response evoked by infusion of ricinoleic acid directly into the proximal small intestine.

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Effect of Deoxycorticosterone Acetate and 16β-Hydroxy-Dehydroepiandrosterone on Blood Pressure and Plasma Renin Activity of Rats[1] (39364)

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The blood pressure response of patients with low renin essential hypertension to drugs which interfere either with the synthesis of adrenocortical hormones, or with the action of these hormones on peripheral target organs, suggests that hypersecretion of an as yet unidentified mineralocorticoid is causative (1). Recently, it has been found that patients having that disorder excrete excessive quantities of a steroid, 16β-hydroxy-dehydroepiandrosterone (16β-OH-DHEA), unlike patients with either nonhypertensive disorders or other forms of hypertension. Studies on the urinary Na/K ratio of adrenalectomized rats indicated that the steroid possessed one-fortieth the mineralocorticoid activity of aldosterone, suggesting that this steroid might be responsible for low renin essential hypertension (1).

In a previous study we reported that neither 1 nor 2 mg/day of 16β-OH-DHEA caused hypertension in rats sensitized to mineralocorticoid hypertension by mononephrectomy and a high salt intake (2). Under this regimen, however, plasma renin activity (PRA) was maximally depressed even in salt-loaded control rats, doubtless as a result of the salt excess (3), thus making it impossible to determine whether the steroid had mineralocorticoid effects on this function.

The present experiment was designed to compare PRA, blood pressure, and organ weight changes in kidney intact rats on a normal salt intake with equipotent mineralocorticoid dosages of deoxycorticosterone acetate (DOCA) and 16β-OH-DHEA.

Materials and methods. Female SPD rats were obtained from ARS Sprague-Dawley Company. They were divided into groups as indicated in Table I. Animals of groups 1, 2, and 3 received, respectively, 3.125 mg of DOCA in sesame oil, 5 mg of 16β-OH-DHEA diacetate in oil, and oil vehicle only by subcutaneous injection once daily. DOCA treatment causes hypertension in rats having both kidneys and on a normal diet (4). However, we have found that regardless of the dosage employed, treatment must be continued for at least 3 weeks in order to obtain a significant incidence of the disorder (unpublished observations). Therefore, the dosage of 16β-OH-DHEA was determined as the maximal daily dosage that could be given for 3 weeks, with the quantity available. The dosage of DOCA was then set at 0.625 of that amount, a quantity greatly in excess of that required to cause hypertension (4), since the steroids have been assigned mineralocorticoid activities, respectively, one-fortieth and one-twenty-fifth that of aldosterone. The animals were individually caged in temperature-controlled quarters and given Purina Laboratory Chow and tap water *ad libitum*.

The systolic pressure of unanesthetized animals was taken on the twenty-third day, using a PE-300 Programmed Electrosphygmomanometer and appropriate modules attached to a DMP-4B Physiograph (Narco Biosystems, Inc., Houston, Texas). Values above 150 mm Hg were regarded as hypertensive.

16β-OH-DHEA was synthesized according to the method of Aoki *et al.* (5). The steroid, obtained as the diacetate, proved to have physical properties identical to an authentic sample obtained from Steraloids and to yield on hydrolysis a compound that was indistinguishable from a sample of 16β-OH-DHEA provided by Dr. Kirk from the Steroid Reference Collection (London).

At the end of each week the rats were individually placed in metabolism cages for 24 hr for urine collection. During this pe-

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TABLE I. Pertinent Findings in Rats Treated with DOCA or 16β-OH-DHEA.

	DOCA	16β-OH-DHEA	Controls
umber of animals	6	7	6
ody wt (g)			
Initial	101 ± 1[a]	102 ± 2	100 ± 2
Final	169 ± 7	183 ± 1	183 ± 3
rine volume (ml/24 hr)			
Week 1	10.7 ± 2.1[b]	5.4 ± 0.9	4.3 ± 0.8
Week 2	7.9 ± 1.2	5.0 ± 0.4	5.8 ± 0.6
Week 3	8.8 ± 0.6	6.1 ± 1.2	7.4 ± 0.7
stolic pressure (mm Hg)	157 ± 10[b]	120 ± 3	128 ± 5
eart rate (/min)	345 ± 15	399 ± 5	368 ± 22
RA	0.08 ± 0.04[b]	1.5 ± 0.3	1.8 ± 0.2
rgan wt (mg/100 g body wt)			
Heart[c]	343 ± 9[b]	265 ± 6[b]	289 ± 6
Kidneys	900 ± 23[b]	744 ± 22	762 ± 27
Thymus	128 ± 20	119 ± 9[b]	143 ± 5
Adrenals	24.6 ± 1.1	25.0 ± 0.9	27.8 ± 1.1

an ± SEM.
ferent from controls ($P < 0.05$).
ntricles only.

ater, but not food, was available.

animals were killed by rapid decapi- on the twenty-fourth day, and trunk was collected within 5 sec into chilled, nized flasks. It was then transferred lastic vials and frozen at −20°, where nained until plasma renin activity) was measured by the method of Pet- *et al.* (6) and expressed as nanograms giotensin generated per milliliter of a per hour of incubation.

lts. Measurement of 24-hr urine out- uring the experiment revealed that -treated rats tended to excrete larger volumes than controls, whereas rats 16β-OH-DHEA did not (Table I).

the twenty-third day, DOCA-treated id become hypertensive, average sys- ressure of the group being 157 ± 10 Ig. This contrasted with 128 ± 5 in ls and 120 ± 3 in those given 16β- HEA. The heart rate varied inversely ne blood pressure, the respective val- ing 345 ± 15, 368 ± 22, and 399 ± 5 min (Table I).

ma renin activity of control rats 1 1.1–2.7 (av. 1.8 ± 0.2) ng/ml/hr. ompared with 0.0–0.3 (av. 0.08 ± in DOCA-treated rats and 0.7–3.1 .5 ± 0.3) ng/ml/hr in rats given 16β- HEA (Table I).

an weights revealed a significant en- ent of hearts and kidneys in DOCA- l rats whereas these organs in rats given 16β-OH-DHEA were slightly smaller than in controls. There were no significant differences between thymus or adrenal weights in any of the groups (Table I).

Discussion. Despite the fact that 3.125 mg of DOCA should have equivalent mineralocorticoid potency to 5 mg of 16β-OH-DHEA, assuming that the respective levels of activity were one-twenty-fifth and one-fortieth that of aldosterone as reported, the former caused hypertension, enlargement of hearts and kidneys, and a reduction of plasma renin activity to undetectable levels, whereas the latter had no such effects.

Our previous studies have shown that 16β-OH-DHEA had no detectable effects at either 1 or 2 mg/day on blood pressure or organ weight of salt-loaded mononephrectomized rats. Funder *et al.* (7) have reported that it has negligible affinity for mineralocorticoid binding sites in the rat kidney. Both Edelman (personal communication) and Higgins (personal communication) have found it to have no effect on sodium transport in the isolated toad bladder. Finally, we have been unable to detect significant mineralocorticoid activity in this steroid in two different rat bioassay systems (<0.1 that of aldosterone).

Taken together, these findings would suggest that 16β-OH-DHEA is essentially biologically inert as a mineralocorticoid. If so, there is little reason to assume that it could directly have anything to do with the genesis of low renin essential hypertension. It could, of course, be a metabolite of some other steroid, presumably of adrenal origin, which has blood pressure elevating properties.

Alternatively, although possessing insignificant mineralocorticoid properties of its own, 16β-OH-DHEA might potentiate the activity of other steroids; such behavior has recently been described for 16α, 18 dihydroxy-deoxycorticosterone, a metabolite of 18 hydroxy-deoxycorticosterone. The metabolite, at a dosage of 10 μg, did not promote sodium retention in adrenalectomized rats, although the same dosage greatly enhanced the sodium retention resulting from the administration of 50 μg of aldosterone. Interestingly, the adrenal glands from patients with low renin essential hypertension converted the precursor into the metabolite under *in vitro* conditions from four to five times more effectively than did control adrenals (8). This raises the possibility that the disease might be caused by steroids which potentiate the activity of mineralocorticoids, rather than playing a direct causative role.

Summary. Intact female Sprague-Dawley rats were given daily injections of either 3.125 mg of deoxycorticosterone or 5.0 mg of 16β-hydroxy-dehydroepiandrosterone, calculated from the activities reported for each to be equivalent mineralocorticoid dosages. The former caused hypertension, cardiorenal enlargement, increased urine output and depressed PRA. Treatment with 16β-OH-DHEA had no such effect, raising questions regarding its classification as a mineralocorticoid.

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hilic and Respiratory Properties of NADH and Succinate Dehydrogenase Sites in Mitochondria from Various Tissues of the Rat (39365)

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ently we showed with the use of lipoprobes (tetraalkylammonium bro-) that the NADH dehydrogenase site ore lipophilic than the succinate dehy- ıase site (1) or other flavin-linked res- ry sites (2) in the inner membrane of er mitochondria. We considered that sponse of these receptor sites to a ilic probe, tetrabutylammonium bro- might be invariant with respect to the of origin since these sites are essential energy metabolism of all aerobic tis- For example, Carafoli and Lehninger owed that another energy-linked proc- alcium accumulation, occurred uni- in mitochondria from diverse tissues ebrates.

ıis communication we report that the ilic (hydrophobic) nature of the H dehydrogenase and succinate dehy- ıase sites differed in mitochondria ed from heart, spleen, liver, kidney, ain; there was no association between e lipophilicity parameters and em- gical origin of the tissues.

rials and methods. Hepatic mito- ia were prepared from male ie-Dawley rats as described previ- (1); the same procedure was used to mitochondria from spleen, heart, and . Brain mitochondria were prepared ing to Ozawa *et al.* (4). Mitochondria ent for a single assay of respiratory l (equivalent to 2.5 mg of mitochon- rotein) were obtained from 0.4 g of 0.1 g of liver, 0.3 g of kidney, 0.4 g of , and 0.9 g of brain. (The method for nitochondria affords a relatively poor but was chosen for its rapidity and nctional quality of the isolated organ- Approximate fresh weights of these in a single 200–250 g rat were: 1 g t, 11 g of liver, 2.5 g of kidneys, 0.8 g en, and 1.7 g of brain. Respiratory rates, respiratory control ratios (RCR), and mitochondrial protein were determined as previously reported (1, 5). Tetrabutylammonium bromide was a product of Eastman Organic Chemicals. Substrates and other biochemicals were purchased from Sigma Chemical Co.

Results and discussion. Variations in respiratory properties were recorded for mitochondria obtained from heart, liver, kidney, spleen, or brain (Table I). For example, using glutamate as substrate in the absence of phosphate acceptor (state 4), the relative rates of oxygen consumption were: brain > kidney > heart = liver = spleen. This ranking referred to the effectiveness of NADH dehydrogenase and it was not determined specifically by the glutamate dehydrogenase activity of the respective tissue. (Unpublished data indicated that the tissue order of activity for glutamate dehydrogenase in the rat was: liver > kidney > heart > brain.) With respect to succinate oxidation during state 4 respiration, the relative rates were: heart = kidney > brain > liver = spleen. Mitochondria from spleen were the most sluggish toward both substrates regardless of metabolic state of the organelles.

Addition of phosphate acceptor stimulated further the oxygen consumption of mitochondria from all tissues (state 3). All mitochondrial preparations were under respiratory control (cf. RCR) thus indicating that structural and functional integrity of their membranes was maintained during isolation. Of the five tissues, kidney was most responsive to addition of ADP during oxidation of both substrates. The relative order of the mitochondrial preparations in this regard was: kidney > heart = brain > liver > spleen.

Tetrabutylammonium bromide, a probe of mitochondrial inner membrane lipophilicity (1, 2), was used to characterize the

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served.

TABLE I. Respiratory Activities of Mitochondria from Various Rat Tissues.[a]

	Tissue				
Activity	Heart	Liver[b]	Kidney	Spleen	Brain
		Glutamate respiration			
State 4	11.5 ± 1.8	10.7 ± 0.6	16.6 ± 1.7	9.5 ± 1.2	20.4 ± 1.3
State 3	72.1 ± 26.1	51.6 ± 4.2	77.0 ± 9.0	32.2 ± 4.8	71.2 ± 6.0
RCR	5.87 ± 1.38	4.86 ± 0.34	4.61 ± 0.08	3.34 ± 0.12	3.48 ± 0.12
		Succinate respiration			
State 4	99.1 ± 11.0	28.0 ± 2.3	92.4 ± 0.6	31.5 ± 1.2	74.1 ± 5.8
State 3	184.6 ± 6.4	99.4 ± 5.7	314.9 ± 4.4	67.7 ± 3.9	157.4 ± 12.9
RCR	1.89 ± 0.13	3.27 ± 0.11	3.41 ± 0.03	2.14 ± 0.05	2.13 ± 0.09

[a] Respiratory rates are nanogram atoms of oxygen consumed per minute per milligram of mitochondrial protein. RCR is the ratio of the respiratory velocity stimulated by ADP to the velocity obtaining on exhaustion of ADP. Means ± SE for at least four preparations. Conditions were those of Fig. 1.

[b] Values for liver are from reference (7).

influence of tissue origin on the lipophilic properties of the membranal sites for NADH dehydrogenase and succinate dehydrogenase. The amphipathic tetraalkylammonium cation functions selectively and reversibly (6) as an inhibitor of phosphorylating oxidation or as an uncoupler, depending on concentration (1). Figure 1 presents the effect of tetrabutylammonium bromide on respiratory control in mitochondria from the different tissues during glutamate oxidation (NADH dehydrogenase) and succinate oxidation (succinate dehydrogenase). An inhibitor concentration that caused a specific depression of RCR was used to estimate the lipophilic character of the respective receptor site (1). The concentration required for 30% depression of RCR, I_{30}, was selected here for comparisons and these values were summarized in Table II from data in Fig. 1.

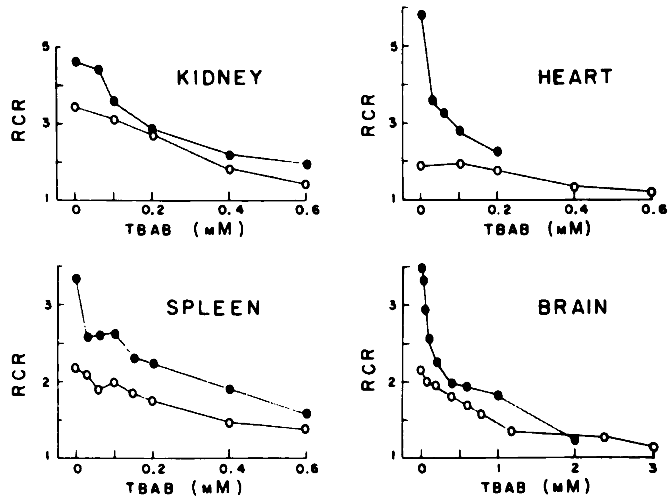

Fig. 1. Depression of respiratory control ratio (RCR) by tetrabutylammonium bromide. Oxygen consumption (nanogram atoms of oxygen per minute per milligram of mitochondrial protein) was determined polarographically at 30° with a Clark fixed voltage electrode. The 3-ml reaction mixture (pH 7.4) contained the indicated concentration of tetrabutylammonium bromide (TBAB) plus 0.33 *M* mannitol, 3.5 m*M* potassium phosphate, 3.5 m*M* KCl, 0.33 m*M* EDTA, 4 mg of dialyzed crystalline bovine serum albumin, 1.4 m*M* L-glutamate (●) or succinate (○), and mitochondria from kidney, heart, spleen, or brain corresponding to 2.5 mg of mitochondrial protein. For brain mitochondria, 4 m*M* substrate and 0.1 *M* KCl were substituted in the reaction mixture for their counterparts. RCR was computed as the ratio of the respiratory velocity in presence of 0.4 μmole ADP (added in 30-μl volume) (metabolic state 3) to the velocity after exhaustion of ADP (state 4). Each data point represents the mean of at least four mitochondrial preparations. The standard error of the mean was equal to or less than the diameter of each data symbol.

: II. THIRTY PERCENT DEPRESSION (I_{30}) BY TETRABUTYLAMMONIUM BROMIDE OF ITOCHONDRIAL RESPIRATORY CONTROL.

sue	NADH dehydrogenase site I_{30}^{a} (μM)	Succinate dehydrogenase site I_{30} (mM)
ırt	25	0.40
:r^{b}	23	0.35
ney	150	0.27
:en	150	0.35
in	200	0.95

concentration of tetrabutylammonium bro- uired for 30% depression of respiratory con- ıg glutamate or succinate oxidation. Condi- e those of Fig. 1.

es for liver are from reference (1).

ptor site was considered more lipo- han another if the concentration re- to inhibit the first was less than that ıry for identical inhibition of the sec- ı. Based on that assumption, the lipo- roperties of the NADH dehydrogen- in mitochondria from heart and liver milar, as were those from kidney and However, the former two were quite ıt from the latter pair. This site was ophilic in organelles from brain and :cinate dehydrogenase site in brain ondria was also the least lipophilic in ues examined. Moreover, lipophilic eristics of the succinate dehydrogen- in organelles from heart, liver, kid- ıd spleen were relatively equivalent 'ariant. Ranking of the tissues by li- city of the NADH dehydrogenase re- site was determined by properties of e per se and not by glutamate dehy- ase susceptibilities of the respective lles since in hepatic mitochondria, for e, indices of lipophilicity were the vhether glutamate, α-ketoglutarate, ydroxybutyrate served as respiratory te (2). On the other hand, receptor r flavin-linked substrates (α-glycero- ate, choline, or succinate) were de- gly lipophilic (2).

relative lipophilic properties of the receptor sites could not be corre- vith the embryological origin of the tissues (i.e. ectoderm, mesoderm, or :rm). The respiratory capacities and lic characteristics of the respective and succinate dehydrogenase sites e related in an as yet undisclosed to metabolic propensities unique to the different organs. Dissimilarities in the lipophilicity of specific inner membranal sites exist in mitochondria from different tissues and those alterations influence associated energy-linked functions. Consequently, estimation of such properties in animals subjected to various experimental conditions and disease states subsequently may permit detection of membrane abnormalities which, in turn, would be of obvious interest from the standpoint of understanding basic mechanisms of disease.

Summary. Mitochondria were isolated from heart, liver, kidney, spleen, and brain of the rat. With overall regard to both resting and activated respiratory velocities with either glutamate or succinate, as well as the respective degrees of respiratory control, kidney mitochondria were most efficient and spleen mitochondria least so. A probe of mitochondrial inner membrane lipophilicity with tetrabutylammonium bromide showed that NADH dehydrogenases from liver and heart were similar, as were also those from kidney and spleen. With the exception of brain, only small differences were observed in lipophilic properties of succinate dehydrogenases from the various other tissues. Variation in lipophilic characteristics of the two sites on the mitochondrial inner membranes could not be correlated with embryological origin of the tissue.

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Effects of Propranolol on Renin Release during Chronic Thoracic Caval Constriction or Acute Renal Artery Stenosis in Dogs (39366)

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The mechanisms which regulate renin release in dogs with chronic thoracic caval constriction (1) or with suprarenal aortic constriction (2-4) have been studied under acute conditions during anesthesia. In dogs with chronic thoracic caval constriction the renal nerves play an important modulatory role in renin release although they are not essential since as much as an eightfold increase in PRA (1) was present after renal denervation. An increase in renin secretion occurred following acute suprarenal aortic constriction in adrenalectomized, anesthetized dogs with a denervated, nonfiltering kidney (4). This latter study eliminated the influence of increased circulating epinephrine but not norepinephrine, and the affects of neither catecholamine were excluded in the studies of the dogs with chronic caval constriction. The present observations with propranolol were made to determine the effect of blockade of the β-receptor mechanism for renin relesae in these two experimental situations.

Materials and methods. Experiment 1. Chronic response to d,l-propranolol in dogs with thoracic inferior vena cava constriction. Six female mongrel dogs weighing from 19-24 kg were placed in metabolic cages and urine was collected daily for determination of renal sodium and potassium excretion. The animals were maintained on a diet containing 60 and 45 mEq/day of sodium and potassium, respectively. Arterial blood pressure and heart rate were measured by percutaneous puncture of the femoral artery with a 22-gauge needle attached to a Statham pressure transducer and recorded on a Hewlett-Packard 7702B recorder. All dogs were started on oral d,l-propranolol (240 mg twice/day) at 8:00 AM and 4:00 PM at least two days before the thoracic inferior vena cava was constricted under sterile conditions to produce experimental ascites (5). Six to 8 days after caval constriction, d,l-propranolol administration was stopped and the dogs allowed to recover. Unless otherwise stated, all blood samples were taken from the jugular vein in the morning 16 hr after 4:00 PM dose of the previous day of d,l-propranolol.

Experiment 2: Acute response to d,l-propranolol in conscious dogs with caval constriction. Six to 8 days after caval constriction, four of the dogs of experiment 1 were brought to the laboratory and their bladders were catheterized and washed with distilled water. A control urine sample (1 hr) and a blood sample for PRA and plasma propranolol determination were taken before the morning dose of d,l-propranolol was administered. Renal sodium excretion, PRA and plasma propranolol levels were measured every hour for 5 hr following propranolol administration. During the experiment the dogs lay quietly on the floor.

Experiment 3: The effects of d,l-propranolol in conscious dogs with acute renal artery constriction. An inflatable cuff was placed around the left renal artery of four female mongrel dogs (19-21 kg) under sterile conditions; a tube extended to the outside of the chest wall. The dogs were allowed to recover for at least 7 days before the acute experiment; they were maintained on a standard diet as described above. For the acute experiment, arterial pressure was measured through an indwelling femoral artery catheter. A saphenous vein was cannulated for infusion of d,l-propranolol. Blood samples for PRA, plasma electrolytes, and plasma propranolol concentration were obtained from either a jugular or a saphenous vein. After the vessels were cannulated, a saline infusion (0.5 ml/min) was begun at least 30 min before the first blood samples were taken. After two control samples (15 min apart), the renal artery cuff was inflated with 0.5-1.5 ml of water to constrict the vessel. Blood samples were taken 30 and 45

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min after constriction. The constriction was then released and the dogs allowed to recover before samples were taken after 60 and 75 min. d,l-Propranolol was then infused at 0.2 mg/kg/hr and a propranolol blood sample was taken after 30 min. The renal artery cuff was inflated to exactly the same degree by use of the same amount of water and samples taken 30, 90, 150, 210, and 270 min during propranolol infusion and renal artery stenosis. Throughout the experiment the animals lay quietly on the floor.

Analytical methods. Venous blood samples for the determination of PRA were placed in chilled test tubes containing 0.1 ml of 10% ethylenediaminetetraacetic acid for each 10 ml of blood. The samples were centrifuged and plasma was frozen until it was prepared for assay by the method of Schneider *et al.* (6). PRA was determined by measuring the pressor response in the pentobarbital-anesthetized, pentolinium-blocked rat in comparison with known amounts of angiotensin II (Hypertensin, Ciba). PRA is expressed as nanograms angiotensin II/ml generated during 3 hr of incubation. Venous blood samples for the determination of plasma electrolytes and propranolol concentrations were obtained and placed in chilled test tubes containing 0.05 ml of heparin per 10 ml of blood and held in an ice bath until centrifuged. The plasmas were decanted and refrigerated until analyzed. Plasma and urine electrolytes were determined by flame photometry. Plasma propranolol levels were assayed by Ayerst Laboratories (Montreal, Canada) by the fluorometric method of Shand *et al.* (7).

Statistical analysis of paired data was performed by Student's *t* test.

Results. Experiment 1: Chronic response to d,l-propranolol in dogs with thoracic inferior vena cava constriction. The response of six dogs to caval constriction during chronic propranolol administration is presented in Fig. 1. In all six animals high circulating levels of plasma propranolol were obtained 3 to 8 hr after the morning dose of the drug. However, 16 hr after the evening dose, plasma levels were low. Heart rate was reduced significantly with propranolol administration from 133 ± 7 (SEM) beats/min to 95 ± 6 beats/min ($P < 0.005$). Heart rate remained low during caval constriction and propranolol treatment (83 ± 8 beats/min) ($P < 0.025$) and during recovery increased to 131 ± 10 beats/min ($P < 0.0125$). These values for heart rate were recorded in the morning 16 hr after the evening dose of propranolol. PRA increased significantly after constriction of the inferior vena cava although d,l-propranolol was administered ($P < 0.025$). During the last two days of caval constriction and propranolol administration, PRA fell toward the control level ($P < 0.05$) but did not reach this level ($P < 0.025$). After d,l-propranolol administration was stopped PRA rose to 20 ± 3 ng angiotensin II/ml ($P < 0.05$). Arterial pressure fell after caval constriction and d,l-propranolol administration from 143 ± 7 to 128 ± 7 mm Hg ($P < 0.01$) and increased during recovery to 145 ± 6 mm Hg ($P < 0.025$). In all animals renal sodium excretion decreased during the second day of thoracic caval constriction and remained low throughout the remainder of the experiment. Ascites formation continued unabated throughout d,l-propranolol administration and caval constriction.

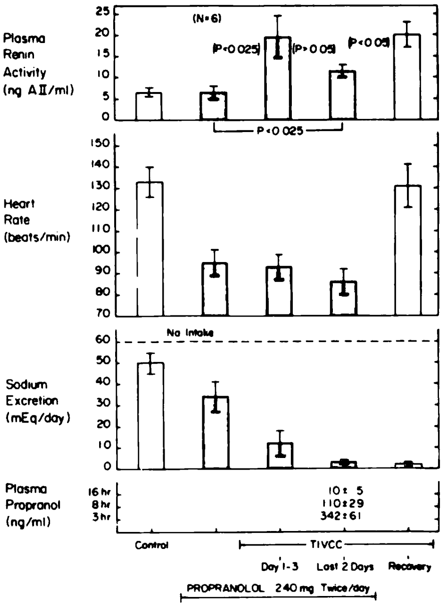

FIG. 1 Chronic response to d,l-propranolol in six dogs with chronic thoracic caval constriction. Abbreviations are same as in Fig. 2.

There was no change in plasma potassium concentration during the administration of d,l-propranolol.

Experiment 2: Acute response to d,l-propranolol in dogs with thoracic caval constriction. Since propranolol reached such low levels 16 hr following the afternoon dose, it was decided to observe the changes in PRA and renal sodium excretion during high levels of circulating propranolol. The experiment was designed to determine if PRA returned to normal and if sodium excretion increased transiently when the circulating level of propranolol was high. During the control observations (18–21 hr after the afternoon dose of d,l-propranolol), the plasma level of propranolol was undetectable in two of the four dogs. During this control period, PRA was 10 ± 2 ng angiotensin II/ml. in comparison with the 80-ng level found in dogs with caval constriction but not receiving d,l-propranolol (1), and renal sodium excretion was low (2–3 μEq/min). PRA and renal sodium excretion were unchanged for 5 hr after a 240-mg oral dose of d,l-propranolol; at 3 and 5 hr the plasma propranolol levels were 210 and 130 ng/ml, respectively.

Experiment 3: The effects of d,l-propranolol on acute renal artery constriction in conscious dogs. The response of four dogs to renal artery stenosis before and during an intravenous infusion of d,l-propranolol is presented in Fig. 2. Before propranolol administration, arterial pressure increased during constriction from 120 ± 3 to 136 ± 6 mm Hg ($P < 0.05$) and decreased during recovery after release of the constriction to 119 ± 4 mm Hg ($P < 0.025$). PRA increased with renal arterial constriction from 6 ± 1 to 12 ± 4 ng angiotensin II/ml ($P < 0.05$) and fell during recovery (6 ± 1 ng angiotensin II/min) ($P < 0.05$). Heart rate fell from 110 ± 10 to 84 ± 10 beats/min ($P < 0.025$) during the first 30 min of d,l-propranolol infusion before reconstriction of the renal artery and remained low during d,l-propranolol infusion. Upon reconstriction of the renal artery during d,l-propranolol, arterial pressure increased from 119 ± 4 to 146 ± 9 mm Hg ($P < 0.025$). Again, PRA more than doubled during renal artery stenosis ($P < 0.005$). Neither the increased arterial pressure nor PRA was attenuated by d,l-propranolol infusion during renal artery constriction. However, during d,l-propranolol infusion before renal artery stenosis PRA decreased significantly from the last recovery period from 5.9 to 4.0 ± 1 angiotensin II/ml ($P < 0.05$). Plasma potassium fell significantly from 4.6 ± 0.1 to 4.1 ± 0.1 mEq/liter ($P < 0.05$) after 5 hr of propranolol infusion, but was unchanged during the first 4 hr.

Discussion. Constriction of the thoracic inferior vena cava in dogs is a potent stimulus for the hypersecretion of renin, hyperaldosteronism, salt, and water retention, and ascites formation (1, 5, 8, 9). Studies during chronic renal denervation (1) have revealed a 50% drop in PRA from a 16-fold to an 8-fold elevation above normal without any detectable change in renal sodium excretion or ascites formation. Also, Witty *et al.* (1) observed an acute decrease in renin secretion during intravenous infusion of propranolol in anesthetized dogs with caval constriction but not to the control level. In the

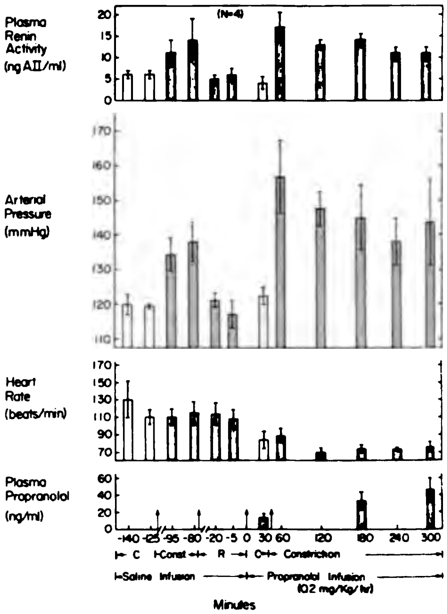

FIG. 2. Acute response to renal artery constriction before and during d,l-propranolol administration in dogs. Abbreviations of C, Const., and R are, respectively, for control, constriction, and recovery. The open bars are for values determined during the control periods.

nt study, chronic daily propranolol ad-
tration failed to block the increase in
in conscious dogs in response to tho-
caval constriction; PRA increased
an average control value of 6 ± 1 ng of
tensin/ml before caval constriction to a
nal level of 23 ± 3 ng angiotensin/ml
0.025) after 4–5 days of propranolol
nistration. These are the first such data
nscious dogs with caval constriction in
actual measurements of plasma pro-
lol have been made and there is evi-
of complete β-blockade. However,
vel of PRA was significantly lower (*P*
001) than the high PRA of 80 ng of
tensin/ml observed previously (1) in
ious intact dogs with caval constriction
d under similar conditions. Neverthe-
during propranolol PRA was high
gh to produce almost complete reten-
of sodium and ascites formation.
a propranolol levels during the first 8
er the morning dose were as high or
r than those observed to produce com-
β-adrenergic blockade (10–12). The
eart rate present before the 8:00 AM
of propranolol also indicates that β-
ade was still present 16 hr after the last
given at 4:00 PM on the previous after-
. In four dogs studied acutely after 6–8
of propranolol administration, the av-
PRA was 10 ± 2 ng angiotensin/ml
e the 8:00 AM dose of propranolol.
findings suggest that β-blockade was
tially complete at 16 hr after the last
PM dose of propranolol from the pre-
day. Also, renal sodium excretion dur-
e 5 hr remained at the low rate charac-
c of dogs with thoracic caval constric-
It should be emphasized that there is
idence that d,l-propranolol exerts a
anesthetic action to block renin release
se the d and l isomers of propranolol
qually potent in stabilizing the cell
rane (13) but only l-propranolol
s renin release (14). These observa-
show that nonadrenergic mechanisms
volved in the hypersecretion of renin
s with chronic caval constriction.

role of the adrenergic nervous system
diating the increase in renin secretion
ing renal artery stenosis was also stud-
he important feature of the experiment is that there was no attenuation by propranolol of the increases in PRA and arterial pressure secondary to renal artery constriction. Plasma propranolol levels increased progressively during the 5-hr infusion to reach an average level of 46 ± 14 ng/ml; this level has been reported previously (10–12) to achieve complete β-blockade. These data, therefore, give no indication that a β-adrenergic receptor mediates the increase in renin release following renal artery stenosis. The findings agree with an earlier report of the effects of renal artery constriction in anesthetized dogs by Winer (15) and a more recent preliminary study in two conscious dogs following renal artery stenosis (16). It seems likely that the increase in renin secretion following renal artery constriction is mediated at least in part by the renal vascular receptor (2, 3). Evidence for involvement of this receptor was provided by Blaine, *et al.* (2, 3) who showed that acute aortic constriction increased renin release in adrenalectomized dogs with a denervated, nonfiltering kidney.

Summary and conclusions. Dogs were given d,l-propranolol before and after thoracic inferior vena cava constriction (TIVCC) or renal artery constriction (RAC) to determine if the increase in plasma renin activity (PRA) and associated biological activity could be suppressed. Oral propranolol administration (240 mg twice daily) was begun at least 2 days prior to TIVCC in six dogs; heart rate was reduced from 133 to 95 beats/min but PRA did not change with propranolol administration. After TIVCC and during continued propranolol administration, daily renal sodium excretion fell from an average value of 50 to less than 3 mEq sodium/day and PRA was elevated two- to fourfold. With prolonged propranolol adminstration during TIVCC, PRA returned toward normal levels but renal sodium excretion remained low. At this time, hourly measurements of sodium excretion showed no change after a 240-mg oral dose of propranolol although very high plasma levels of propranolol were achieved; also, PRA was unchanged and low. The effects of RAC were studied before and during an intravenous propranolol infusion (0.2 mg/kg/hour) in conscious animals. Before propranolol administration, arterial pressure increased during RAC from 120 to 136 mm Hg and PRA doubled. Propranolol infusion lowered heart rate from 110 to 84 beats/min, but arterial pressure and PRA were not attentuated by propranolol during RAC. The data indicate that non-beta-adrenergic mechanisms are involved in renin release during TIVCC and RAC.

The authors wish to thank Mr. Henry L. LeMien, Jr., of Ayerst Laboratories for the supply of propranolol and for help in the analysis of plasma propranolol.

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GS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 229-231 (1976)

t of Nonsuckling Interval in the Ether-Induced Release of Immunoprecipitable Prolactin into the Plasma of the Lactating Rat (39367)

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concentration of prolactin in the a quickly rises following the onset of inhalation in the nonlactating rat (1–8, h see 9). The plasma concentration, er, seldom exceeds 50 ng/ml and is ed for only a few minutes. Prolonged anaesthesia has no effect upon plasma tin levels (6). In the lactating rat, the a prolactin concentration following inhalation exhibits a pattern similar to btained in nonlactating rats, but con- markedly with the 200–300 ng/ml appear in the plasma after 10–12 min kling (10–15) and which can be main- for periods up to 90 min by continued ng (14–15).

e the release of prolactin by suckling modified by a variety of factors (16) of interest to determine which factors modify the release of prolactin in re- to stress in the lactating rat. In the t study, we have analyzed the influ- f the nonsuckling interval.

erials and methods. Primiparous lac- rats of the Holtzman strain were d in individual cages in a room main- at 23–25° and with alternating 14-hr 0-hr dark periods. They were given a -casein-sucrose diet to which 5% cel- was added for fecal bulk; tap water vailable *ad libitum*. Litters were ad- to eight pups on Days 1–2 postpar- nd to six pups on Day 4 postpartum. others were handled periodically dur- e lactation period.

experimental manipulations of the ckling interval were made prior to ap- on of an ether stress. (i) The pups removed on the morning of Day 14 e mothers were subjected to ether either 8 or 12 hr later. (ii) The pups emoved the morning of Day 13. Dur- e next 12 hr, they were returned to nothers either for a single 10-min pe- f suckling (at the end of the twelfth hour), for two 10-min periods of suckling (at the sixth and twelfth hours), or for three 10-min periods of suckling (at the fourth, eighth, and twelfth hours). The pups were discarded at the completion of the twelfth hour suckling and, after a subsequent 12-hr period allowed to standardize pituitary prolactin levels, the mothers in each groups were subjected to ether stress.

Ether stress. Ether (anesthesia grade) was placed in a wide-mouth glass jar (22 × 25 cm) which was covered and placed on a lab stool close to the cage of the mother to be tested. Approximately 5 min later (the 5 min to allow for the atmosphere in the jar to become heavily saturated with ether fumes) the rat was gently removed from her cage and placed into the jar. The animals were etherized in this manner in groups of three to four rats at a time. Once the rats became unconscious (usually within 1 min) they were removed from the jar, quickly guillotined and the trunk blood collected in cooled test tubes. The plasma was separated and kept frozen until later assayed for prolactin. Control mothers (litters removed 6 hr earlier) were gently removed from their cages and swiftly guillotined.

Radioimmunoassay of prolactin. Prolactin in plasma was determined in duplicate 25-μl samples by the NIAMDD radioimmunoassay system (See 14, for details). Mean differences in plasma prolactin concentration between groups were judged significantly different (using Student's *t* test) if the probability was 5% or less.

Results. In the first experiment (Fig. 1A), the concentration of prolactin in the plasma of lactating rats averaged 74 ng/ml (46–160 ng/ml range) following application of acute ether stress after an 8-hr period of nonsuckling. When ether stress was applied after a 12-hr period of nonsuckling, the plasma level of prolactin was significantly lower and averaged 24 ng/ml (18–40 ng/ml range).

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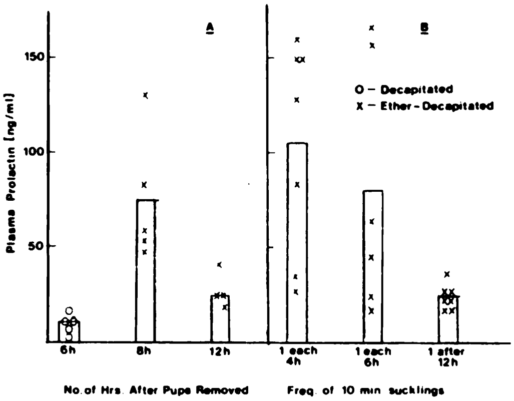

FIG. 1. Prolactin concentration in plasma of lactating rats following acute exposure to ether before decapitation. (A) Rats nonsuckled for either 8 or 12 hr prior to ether exposure. (B) Rats suckled, one, two, or three times, each time for 10 min during an initial 12-hr nonsuckling period; all rats then were exposed to ether 12 hr after the last suckling. Bars represent mean values; each symbol represents a single rat.

In the second experiment (Fig. 1B), the plasma prolactin concentration following acute ether stress increased significantly from 24 ng/ml (17–36 ng/ml range) to 104 ng/ml (26–160 ng/ml range) as the frequency of 10-min sucklings was experimentally altered during a 12-hr period from once at the end of the 12-hr period to once every 4 hr. The intermediate frequency of suckling once every 6 hr resulted in an average plasma prolactin concentration of 79 ng/ml. The range of values in this group, however, overlapped with those of the other two groups and thus the mean concentration was not significantly different from that of either of the other two groups. All groups exposed to ether had mean plasma prolactin concentrations which were significantly elevated over that of rats isolated for 6 hr and similarly killed but without being exposed to ether (Fig. 1).

Discussion. Various factors can influence the degree to which the concentration of prolactin increases in the plasma following stress. Neil (1) found that a consistently higher plasma prolactin concentration occurred each day throughout the estrous cycle following ether–laparotomy stress, in comparison with that found in plasma of decapitated rats, except on the afternoon of proestrous when ether–laparotomy failed to increase the already elevated levels. Reir *et al.* (7) demonstrated that in rats treated for 6 days with estradiol the serum prolactin level resulting from ether–laparotomy stress was raised, whereas, following treatment with progesterone it was lowered. Progesterone also reduced the estrogen-stimulated elevation in prolactin levels provoked by ether–laparotomy, an effect previously shown by Chen and Meites (17). Serum prolactin levels resulting from ether inhalation were shown to increase twofold in male and female rats when they were handled twice daily the previous 4 days (2). It is possible, however, that handling in this instance constituted an additional stress.

In the present investigation the plasma prolactin concentration in the lactating rat was significantly more elevated when ether was inhaled after 8 hr than after 12 hr of nonsuckling. When the suckling frequency during a 12-hr period was increased in groups of rats from one 10-min suckling at the end of the twelfth hour to a 10-min suckling once every 4 hr and the rats then exposed to ether 12 hr after the last suckling, we observed the same relationship between length of the nonsuckling interval and the rise in plasma prolactin in response to ether, i.e., the shorter nonsuckling interval resulted in the more elevated prolactin concentration. Prolactin concentration in the anterior pituitary is approximately the same after nonsuckling intervals of 4, 8, and 12 hr (16) so a difference in available prolactin would not appear to be involved in the explanation of these differences. Rather it would appear that more frequent activation of the prolactin releasing mechanism by suckling in some way facilitates the subsequent release of prolactin in response to ether. A central serotinergic mechanism has been implicated in both the suckling-induced (13) and ether-induced (18) release of prolactin. The mechanism of release of prolactin by suckling also contains a hypothalamic dopaminergic system (19, 20) which appears not to be implicated in the ether-induced release (7, 18). Thus, the fact that the frequency of suckling in some way facilitates the release of prolactin in response to ether as well as that in response to suckling (16) suggests the serotinergic link may be common to both mechanisms.

Summary. The concentration of prolactin n the plasma of lactating rats increased rom 24 to 74 ng/ml following acute ether tress as the length of the preceding period of nonsuckling diminished from 12 to 8 hr. n a second experiment the prolactin concentration increased from 24 to 79 to 104 ng/ml as the frequency of 10-min sucklings during a 12-hr period was altered from once at the end of the twelfth hr to once every 6 hr to once every 4 hr and the mothers then exposed to ether 12 hr after the last sucking. These results indicate that more frequent activation of the prolactin-releasing mechanism facilitates the subsequent release of prolactin in response to ether stress.

We are grateful to the NIAMDD Rat Pituitary Hormone Distribution Program for the rat prolactin radioimmunoassay kit, and to Mrs. Tanya McGee for echnical assistance.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 232-236 (1976)

Studies on Rats with Islet Beta Cell Tumors Induced by Nicotinamide and Streptozotocin (39368)

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Rakieten *et al.* (1) first reported that the combined administration of streptozotocin and nicotinamide resulted in the induction of pancreatic islet tumors containing high concentrations of immunoreactive insulin (IRI). It was felt that this rat islet adenoma may provide a suitable experimental model for human insulinoma. This report details observations on the physiologic responses of rats following tumor induction, and on the insulin biosynthetic activity of the tumors. A portion of this work has appeared in abstract form (2).

Methods and materials. Young Holtzman rats weighing 180 g were fasted overnight and injected ip with nicotinamide (350 mg/kg body weight in 0.85% saline). Ten minutes later a single iv injection of streptozotocin (65 mg/kg body weight in a citrate buffer, pH 4.5) was administered, followed by another ip dose of nicotinamide 180 min after the first dose, as described by Rakieten *et al.* (1). The control rats were treated with nicotinamide and citrate buffer. All the animals were weighed weekly and bled monthly by tail vein. Blood glucose was determined with an AutoAnalyzer by a modification of the method of Hoffman (3).

Glucose tolerance test (GTT). The animals were fasted overnight and 30% glucose solution in 0.85% saline was administered by tail vein (1 ml per 100 g body weight). Blood sugar values were determined on samples withdrawn before and ½, 1 and 2 hr after glucose injection. The Diabetic Index (DI), which determined the ability of the animals to handle sudden glucose influx, was calculated by the following equation of Coupland *et al.* (4):

$$\mathrm{DI} = \frac{\text{blood sugar at 1 hr} \times \text{blood sugar at 2 hr of experimental animals}}{\text{blood sugar at 1 hr} \times \text{blood sugar at 2 hr of control animals}}$$

The animals were anesthetized with Pentosol (Sodium pentabarbital 50 mg/ml, Invenex, California), and bled by cardiac puncture. The abdomen was opened and the islet tumors were decapsulated, weighed, and cut into small pieces for various studies.

Effect of glucose load on serum insulin levels of rats bearing beta cell tumors. Another group of approximately 1-yr-old rats exhibiting persistent hypoglycemia (which were found to have tumors on sacrifice) were lightly anesthetized with ether and bled from the orbital sinus. The animals were injected intravenously with 30% glucose solution (1 ml/100 g body weight) and were bled again 30 min later. The insulin levels were determined in 0.1-ml serum samples using an insulin assay kit ("Phadebas Insulin Test" from Pharmacia AB, Uppsala, Sweden).

Insulin biosynthesis in vitro. Decapsulated tumor samples were cut into approximately 2-mg pieces, and pooled samples weighing between 10 and 40 mg were incubated with shaking (30 oscillations per min) at 37° for 1 or 3 hr in media containing either 60 (3.3 m*M*) or 300 mg/100 ml (16.5 m*M*) glucose, and L-[U-^{14}C]leucine, 311 mCi/mmole, 48 μmole/liter (New England Nuclear Inc.). The medium consisted of 1 ml of Krebs Ringer bicarbonate buffer (KRB) with bovine serum albumin (200 μg), ovomucoid trypsin inhibitor (1 μg), and 19 common amino acids excluding leucine (20 μg each), and was equilibrated with 95% O_2–5% CO_2 gas mixture. Following incubation, tissue and medium were homogenized in the cold in AA glass Teflon grinders (Kontes Glass Co., Vineland, N.J.). Trichloroacetic acid (TCA) was added to a concentration of 10%, and the insoluble material was separated by centrifugation and washed twice with 10% TCA. Insulin and proinsulin biosynthesized during incubation were determined as described recently (5).

Elution profiles of bovine serum albumin,

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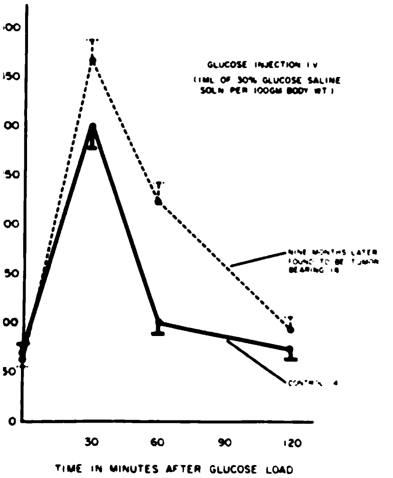
nsulin, and bovine insulin were used tify radioactive proteins in column . Ten-microliter aliquots of 1-ml col- actions were assayed for immuno- : insulin by a double antibody l (6), using rat insulin standards. Pro- ıtents were estimated by the method ry *et al.* (7).

'ts. Glucose tolerance test (GTT). 3 weeks the animals treated with ımide and streptozotocin exhibited a change in their ability to handle a ucose load (Fig. 1).

mean blood glucose level of these increased from 61 to 369 mg/100 ıin 30 min following the glucose in- and was 225 and 96 mg/100 ml 60 0 min after glucose load, respec- By contrast in the control rats an ıl glucose load resulted in an eleva- the blood sugar levels from 60 mg/ at 0 hour to 305, 100, and 74 mg/ after 30, 60, and 120 min, respec- The differences at 30 and 60 min ghly significant ($P < 0.01$). About 8 after the combined drug treatment a umber of rats exhibited persistent 'cemia. The nonfasting blood sugar these animals was about 20 to 25 mg/100 ml lower than that of the corresponding controls. At the time of sacrifice (about 10 months after the combined drug treatment) all the animals with abnormal GTT exhibited sizable islet beta cell tumors.



. Abnormal glucose tolerance in rats 3 weeks tment with streptozotocin and nicotinamide; imals exhibited beta cell tumors after 9 'he differences in the blood sugar levels at 30, 20 min between the control and drug-treated 're significant, $P < 0.01$, < 0.0001 and espectively.

A group of animals exhibiting persistent hypoglycemia (which on sacrifice exhibited beta cell tumors) was administered a glucose tolerance test (GTT), 9 to 10 months after combined drug treatment. As shown in Fig. 2, two distinctly different patterns of blood glucose curves were observed as compared to the control animals. The blood glucose levels of the control rats following the glucose load were 83, 299, 126, and 111 mg/ 100 ml at 0, 30, 60, and 120 min, respectively. By contrast, one group of animals exhibited a tolerance curve typical of a mildly diabetic or subdiabetic animal. The corresponding blood glucose values were 55, 386, 287, and 114 mg/100 ml. The difference from the control rat was highly significant ($P < 0.005$) at 60 min. The beta cells in the islet tumors of these animals perhaps responded sluggishly to the glucose load and hence they were termed "slow acting tumors" (SAT).

The blood sugar levels of the other group were 60, 135, 86, and 77 mg/100 ml, respectively, following glucose administration. The blood sugar levels at 30 and 60 min were markedly lower than those of the

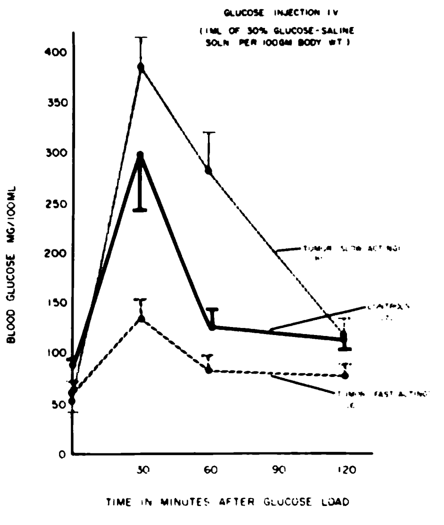


FIG. 2. A comparison of glucose tolerance between normal rats and those bearing beta cell tumors as confirmed by autopsy.

normals at corresponding time periods ($P <$ 0.005 and <0.02, respectively).

Immuno-Reactive-Insulin (IRI) in the serum of rats with beta cell tumors. Table I shows that sera collected from rats exhibiting tumors at autopsy could be classified into two distinct categories on the basis of the immunoreactive insulin content (IRI). One group had high IRI content, while in the other the IRI level was normal.

Effect of glucose load on serum insulin levels of beta cell tumor-bearing rats. Table II indicates that the average serum insulin level of rats was elevated from 44 to 73 μU/ml within 30 min following glucose administration. Only three animals failed to show a positive response to the glucose load. At autopsy all these animals exhibited tumors and, hence, data on these three animals have been included for averaging in the table. By contrast, the serum insulin level in five normal controls was elevated from 29 to 38 μU/ml.

Insulin biosynthesis in vitro. The distribution of immunoreactive insulinlike activity following gel filtration is depicted in Fig. 3A. The major insulin region (I) corresponds to a material having a molecular weight of approximately 6000 daltons (monocomponent insulin), while the secondary region (proinsulin) of approximately 9000 daltons had less immunoreactive insulinlike activity. Following 1 hr of incubation, radioactivity was incorporated into the proinsulin (P) region, while none appeared in the insulin region (data not shown). After 3 hr of incubation, some radioactivity also appeared in the immunoreactive insulin region (Fig. 3B). Further evidence for the biosynthetic relationship of these radioactive proteins was shown by tryptic hydrolysis of the isolated proinsulin to material migrating with monocomponent insulin (Fig. 3C). Polyacrylamide gel electrophoresis of the isolated and separated P and I pools of gel filtration eluates showed that the major radioactive protein of the proinsulin (P) region migrated with an R_f of 0.60, while the radioactivity of the insulin (I) region is represented by two (more electronegative) proteins with R_fs of 0.71 and 0.90 (data not shown).

TABLE I. Serum Immuno-Reactive Insulin (IRI) in the Rats with Beta Cell Tumors.

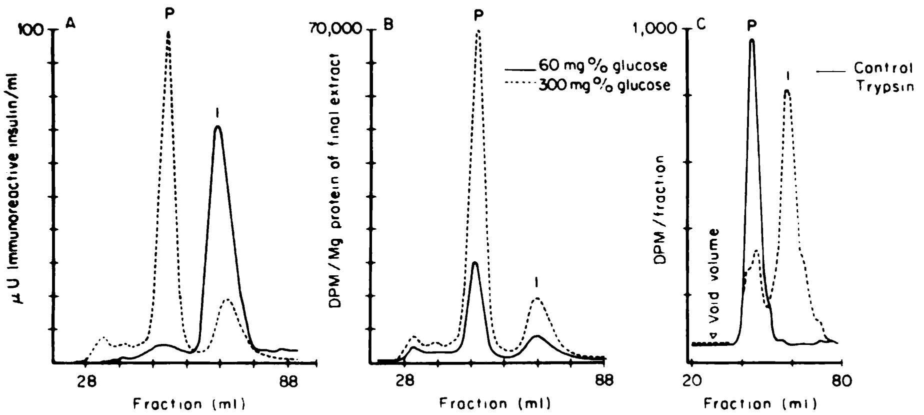
High IRI group		Normal IRI group	
Number of rats	μunits/ml SEM	Number of rats	μunits/ml SEM
6	124 ± 12.7 (76 to 158)	9	40 ± 2.8 (27 to 50)

Incubation of tumor tissue in the medium containing 300 mg/100 ml glucose stimulated the incorporation of leucine into both proinsulin and insulin regions of gel filtration eluates, when compared with incubation in medium containing 60 mg/100 ml glucose (Fig. 3B). An experiment involving three adenomata derived from different animals, in which samples of each tumor were incubated at either high or low glucose concentration, is summarized in Table III. Although there was considerable variation in rates of basal and glucose-stimulated leucine incorporation into proinsulin and insulin in the three adenomata, all of them exhibited a response to elevated glucose levels.

Discussion. Multiple pancreatic islet cell tumors were produced in approximately 85% of the rats which survived 8 months or more after the administration of streptozotocin and nicotinamide. This figure compares favorably with the 64 and 49% rates reported by earlier workers (1, 8). Some animals exhibited hypoglycemia within 8 months following drug administration indicating that islet cell tumors may already have been induced before that time.

TABLE II. Effect of Glucose Administration to Tumor Bearing and Control Rats on the Serum Immuno-Reactive Insulin (IRI) Levels.

		Insulin per ml serum following glucose injection (1 ml of 30% glucose solution per 100 g body weight)			
Condition of rats	Number	0 Min μU/ml SEM	30 Min μU/ml SEM	Difference	P
Tumor	15	44.0 ± 4.3	72.6 ± 9.0	28.6	<0.005
Control	5	29.4 ± 3.3	38.0 ± 5.4	8.6	N.S.



G. 3. (A) Immunoreactive insulin in gel filtration eluate of rat adenoma, solid line. Final Extract (1:40,000 ion). Tissue was incubated 3 hr in KRB containing 300 mg/100 ml glucose. The dashed line represents the poration of [^{14}C]leucine into proteins of the same Final Extract. P = proinsulin region of eluate; I = insulin n. (B) Representative gel filtration eluates of Final Extracts from Adenomas incubated in either 60 or 300 00 ml glucose. (C) The effect of trypsin (1 μg) on isolated proinsulin (P) region (about 1 mg protein) of an oma incubated with [^{14}C]leucine. The control was an equal aliquot of isolated P which received no tryspin ment. The void volume, P and I regions were determined by calibration of columns with appropriate lards.

ABLE III. *In vitro* L-[^{14}C]LEUCINE INCORPORATION INTO PROINSULIN (P) AND INSULIN (I) BY RAT ISLET NOMA IN THE PRESENCE OF LOW (60 mg/100 ml) OR HIGH (300 mg/100 ml) GLUCOSE IN THE MEDIUM.^{a}

	60 mg/100 ml glucose			300 mg/100 ml glucose		
	P	I	I/P	P	I	I/P
Adenoma 1	57,000	26,500	0.47	187,000	61,800	0.33
Adenoma 2	73,800	28,900	0.39	119,000	52,700	0.44
Adenoma 3	346,000	38,300	0.11	681,000	89,000	0.13

Tissue pieces from three adenomas were incubated concurrently for 3 hr in KRB with either the low or high se concentration, and extracted in parallel. Results are expressed as disintegrations per minute per milligram otein of the final extract.

he abnormal glucose tolerance curve 3 ks after the treatment with the com- d drugs, may indicate that although the nals did not exhibit manifest diabetes, were unable to handle a sudden large l of glucose. The DI of these rats was . According to Coupland *et al.* (4) a DI xcess of 1.7 is indicative of subdiabetic . All the rats with subdiabetic DI devel- d pancreatic islet tumors. This observa- suggests some relationship between nic exhaustion of the beta cell and its rplasia and eventual tumor develop- t.

nimals whose glucose tolerance indi- d that they had slow acting tumors had a of 2.34 while animals with fast acting ors had a DI of 0.47.

e serum immunoreactive insulin levels ats with tumors were either high (124 μU/ml) or normal (40 μU/ml) (Table 1). On the basis of serum IRI levels the rats could not be designated as containing fast or slow type of tumors. All the rats with tumors were capable of responding to increased blood glucose levels as shown by the fact that their insulinogenic response to glucose was greater than that of the corresponding control rats (Table 2). The elevation of serum IRI level in controls at 30 min following glucose administration was not significant. Perhaps IRI measurements at earlier time periods may have been more meaningful.

Insulin biosynthetic activity of induced adenomata was demonstrated by means of the *in vitro* incorporation of [^{14}C]leucine into proinsulin and insulin. [^{14}C]proinsulin was readily converted to an insulinlike molecule by trypsin treatment (10, 11). The migration of these proteins on gel electrophoresis closely paralleled those isolated from islets of Langerhans obtained from normal rats by the collagenase digestion procedure (5).

On the basis of radioimmunoassay, monomeric (6000 daltons) insulin represented the major type of immunoreactive insulin in one adenoma (Fig. 3A). Protein material migrating in the 9000-dalton range mostly consisted of proinsulin, and accounted for less than 10% of the immunoreactive insulin in this adenoma. Gel filtration eluates of two other adenoma extracts were also assayed and showed essentially the same pattern of immunoreactivity.

Creutzfeldt *et al.* (11) reported an average tumor insulin content in human adenomata of 24 U/g (0.01–199 U/g). In the rat adenomata reported here, the average IRI content was 412 U/g (165–729 U/g) suggesting that the tumors have large stores of insulin. By contrast, normal rat islets contain 200 U/g of insulin (12).

The ratio of [^{14}C]insulin to [^{14}C]proinsulin, which estimates the extent of conversion of precursor to insulin, was the same at basal (60 mg/100 ml) and elevated (300 mg/100 ml) glucose concentrations, suggesting that enzymatic conversion of proinsulin to insulin is not a limiting step in insulin production by the induced adenomata. In all tumor studies, the elevated level of glucose in the medium (300 mg/100 ml) caused an increased rate of [^{14}C]leucine incorporation into (pro)insulin when expressed on the basis of specific activity (disintegrations per minute per milligram of protein in the extract).

Summary. Islet beta cell adenomata were induced in rats by combined treatment with nicotinamide and streptozotocin. Three weeks after treatment marked alterations in glucose tolerance were noted in animals which later exhibited large beta cell tumors. Eight months after treatment, the rats known to have beta cell tumors on the basis of marked hypoglycemia and later confirmed by autopsy showed variable response to a glucose load. Some tumor-bearing rats showed fast response to glucose load, their blood sugar levels were elevated moderately and returned to normal or below normal levels rapidly; these animals are described as having "fast-acting tumors." Rats with "slow-acting tumors" responded sluggishly to a glucose load; their blood glucose pattern was similar to that of subdiabetic animals. Animals with beta cell tumors exhibited elevated serum insulin levels 30 min after glucose administration.

Insulin biosynthesis by beta cell adenomata was demonstrated by *in vitro* incorporation of [^{14}C]leucine into proinsulin and insulin. In the small number of tumor samples studied, a stimulatory effect of glucose on insulin biosynthesis was observed.

Our grateful thanks to Dr. William E. Dulin, The Upjohn Co., Kalamazoo, Michigan, for the gift of streptozotocin, and to Mr. Steven Koziol of Pharmacia Lab, Piscataway, New Jersey, for Phadebas Insulin Test Kits. The Minnesota Medical Foundation aided this research.

This paper is dedicated to the memory of the late Dr. Arnold Lazarow.

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GS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 237-241 (1976)

The Purification of Calcium-Binding Protein from the Uterus of the Laying Hen (39369)

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stinal calcium-binding proteins from
l species, including the chick (1-3),
, and monkey (5) have been shown to
›endent upon vitamin D for their for-
ı, and a large body of evidence pres-
:xists suggesting a role for these pro-
n the vitamin D-mediated intestinal
ort of calcium. Proteins similar or
:al to the chick intestinal calcium-
g protein have also been detected in
ick kidney (2) and brain (6) and in the
of the laying hen (7). The hen uterine
n-binding protein has been shown to
›endent upon vitamin D_3 for its forma-
7) and to increase in concentration in
sue with the onset of egg-laying and
ise in concentration with its cessation
nd has been localized in the tubular
cells of the shell gland (9). Unlike the
nal calcium-binding protein, the uter-
otein does not appear to be influenced
g-term dietary calcium-restriction (8).
ecent report (10) suggested that the
e calcium-binding protein might be
:nt from the intestinal protein, al-
ı earlier studies, by another group
ıdicated that the two had similar elec-
›retic mobilities. In the present study,
e calcium-binding protein was isolated
ıme of its properties compared to puri-
ıtestinal calcium-binding protein, in-
g immunological and electrophoretic
rties and amino acid composition. The
rities of these characteristics indicate
ıe two proteins are identical.

hods and materials. Hen uterus was
ed from normal mature White Leg-
ıens in lay. Animals were sacrificed by
ıl dislocation, the uterus was excised,
ıe latter homogenized in Tris buffer
'e, pH 7.4 (1:2, w/v), containing
β-mercaptoethanol. Homogenization
l successive steps were conducted at
: homogenates were then centrifuged at 23,000 *g* (max.) for 45 min and the supernatant solutions retained.

The original supernatant solution was subjected to $(NH_4)_2SO_4$ fractionation by the addition of 48 g of solid $(NH_4)_2SO_4$ per 100 ml supernatant solution with vigorous stirring. Mixing was continued for 2 hr following the last addition, and the resulting suspension centrifuged as described before. The $(NH_4)_2SO_4$-soluble supernate (700 ml) was dialyzed exhaustively against Tris buffer and concentrated to 23 ml by membrane ultrafiltration, using an Amicon Model 2000 Diafiltration unit equipped with a UM-10 Diaflow membrane (Amicon Corp., Lexington, Mass.).

Ten milliliters each of the original mucosal supernatant solution and concentrated $(NH_4)_2SO_4$-soluble supernate were applied separately to a 2.5 × 87-cm calibrated Sephadex G-100 column and eluted with Tris buffer mixture. Columns were run in the upward direction by gravity flow with a buffer flow rate of 0.5 ml/min. Three-milliliter (60 drop) fractions were collected. The column calibration procedure has been described previously (11). Fractions from gel filtration column chromatography of the $(NH_4)_2SO_4$-soluble supernate that contained calcium-binding activity and exhibited only several bands on analytical acrylamide gel electrophoresis were pooled and concentrated to 1.0 ml by membrane ultrafiltration and subjected to preparative gel electrophoresis. The procedures for analytical and preparative acrylamide disk gel electrophoresis have been described in detail previously (11).

Column fractions were assayed for total protein concentration by the procedure of Lowry *et al.* (12) and all values expressed on the basis of bovine serum albumin standard solutions.

Calcium-binding activity was assayed by

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:served.

the Chelex-100 ion-exchange procedure of Briggs and Fleischman (13) as modified by Wasserman *et al.* (3).

Double immunodiffusion analyses were conducted in a humidified chamber at room temperature on micro Ouchterlony plates prepared with 1.5% agar (special Agar-Noble, Difco Laboratories, Detroit, Mich.) in barbital buffer (pH 8.6; ionic strength, 0.075). Preparation and demonstration of the specificity of the antiserum have been described previously (14). Twenty-microliter aliquots of both sample and specific antiserum were applied to the agar wells and diffusion allowed to continue for 24 hr.

Immunoelectrophoresis was performed by first subjecting samples to analytical discontinuous gel electrophoresis in 10% acrylamide. At the completion of electrophoresis, gels were slit longitudinally and one-half of each gel embedded in 1.5% agar on a microscope slide. A slot was then cut in the agar parallel to the gel and specific antiserum added. Diffusion was allowed to continue for 24 hr at room temperature in a humidified compartment.

The radial immunodiffusion procedure of Mancini *et al.* (15) as modified by López and Golder (16) was employed for quantitative immunoanalysis of calcium-binding protein in some instances. Specific antiserum was dissolved in 1.5% agar (in barbital buffer, pH 8.6) at a concentration of 4% and 0.5 ml of the mixture delivered to cylindrical wells (4-mm deep × 14-mm diameter) and allowed to harden. Three-millimeter holes were cut in the center of each well and a 20-μl sample placed therein. Diffusion was allowed to proceed for 24 hr, after which the diameter of the precipitin rings were measured. Concentration of calcium-binding protein was determined from a semi-log plot of standard protein solutions.

Amino acid analyses were conducted essentially according to the method of Moore and Stein (17) with the aid of a Beckman model 120 C amino acid analyzer. Approximately 0.5 mg of lyophilized calcium-binding protein was hydrolyzed in 6 *N* HCl, under vacuum, at 110° for 22 hr. HCl was removed by rotary evaporation at 40° and samples were brought to 1.1 ml with pH 2.2 sample-diluting buffer. One-half-milliliter aliquots were applied in duplicate to both the long and short columns for analysis.

Results and Discussion. The results of gel filtration column chromatography on the original mucosal supernatant and the $(NH_4)_2SO_4$-soluble supernatant solutions from hen uterus are shown in Fig. 1. Included for comparison is a chromatographic elution profile of the original supernatant solution from chick intestinal mucosa obtained under identical conditions. On the basis of previous column calibration runs, a V_e/V_0 ratio of 2.0 was obtained for each of the sample runs in Fig. 1, corresponding to a molecular weight of about 28,000. As indicated above, the calcium-binding profile for the original chick intestinal supernate and the concentrated uterine $(NH_4)_2SO_4$-soluble supernate were easily obtained by means of the Chelex-100 ion exchange procedure. However, the method lacked sufficient sensitivity when applied to the original uterine supernatant solution due to the low concentrations of protein therein, as verified by quantitative measurements of calcium-binding protein concentration by radial immunodiffusion. Therefore, the elution profile of original uterine supernate was determined by the use of the sensitive radial immunoassay procedure. With this procedure, it was clearly shown that the column fractions containing uterine calcium-binding protein eluted in essentially the same volume as the calcium-binding activity peak from chick intestinal supernate and the concentrated $(NH_4)_2SO_4$-soluble preparation from the hen uterus.

Bar and Hurwitz (10) have recently reported a slightly greater molecular size for the uterine protein as compared to the intestinal calcium-binding protein on the basis of SDS acrylamide gel electrophoresis. In the present study, estimation of molecular size by gel filtration column chromatography showed essentially no difference in molecular size for the two proteins. Both were estimated to be approximately 28,000 daltons, a value which has been confirmed previously for the chick intestinal calcium-binding protein by gel filtration (3, 18), SDS acrylamide gel electrophoresis (19), and amino acid compositional studies (18, 19). Furthermore, on SDS acrylamide gel elec-

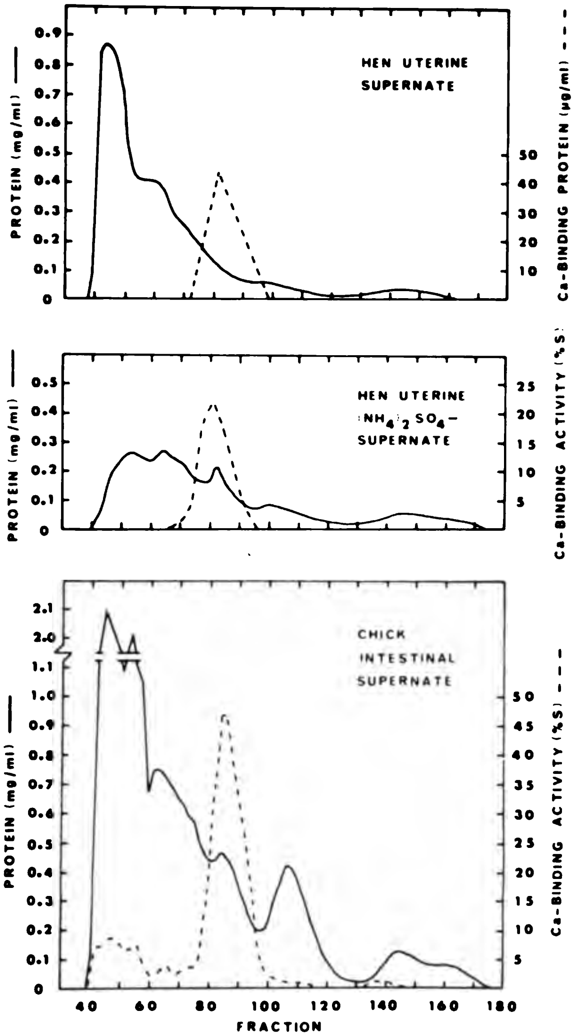

FIG. 1. Separation of original and $(NH_4)_2SO_4$ soluble proteins from hen uterus by gel filtration on Sephadex G-100. Included for comparison is a similar elution profile run on original supernatant solution from chick intestinal mucosa. (●——●), Total protein; (O- - -O), Ca-binding activity.

trophoresis, the hen uterine and chick intestinal calcium-binding protein migrated coincidentally on a single gel, again suggesting identity.

Double immunodiffusion analyses of purified hen uterine calcium-binding protein and intestinal calcium-binding protein are shown in Fig. 2. Immunological identity of the two proteins is verified by total coalescence of the precipitin lines resulting from each. From the immunoelectrophoresis studies (Fig. 3), the location of the precipitin arc in the embedded gels, and the protein bands in the stained gels showed that

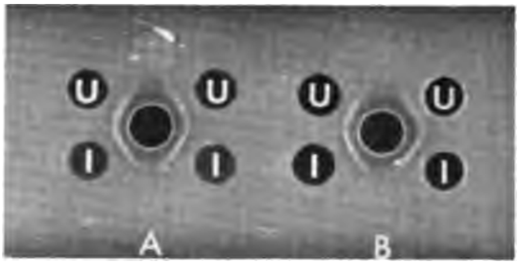

FIG. 2. Double immunodiffusion analysis performed on the original supernatant solutions (A) and purified calcium-binding proteins (B) from hen uterus (U) and intestine (I). Diffusion against antiserum specific for chick intestinal calcium-binding protein (center wells).

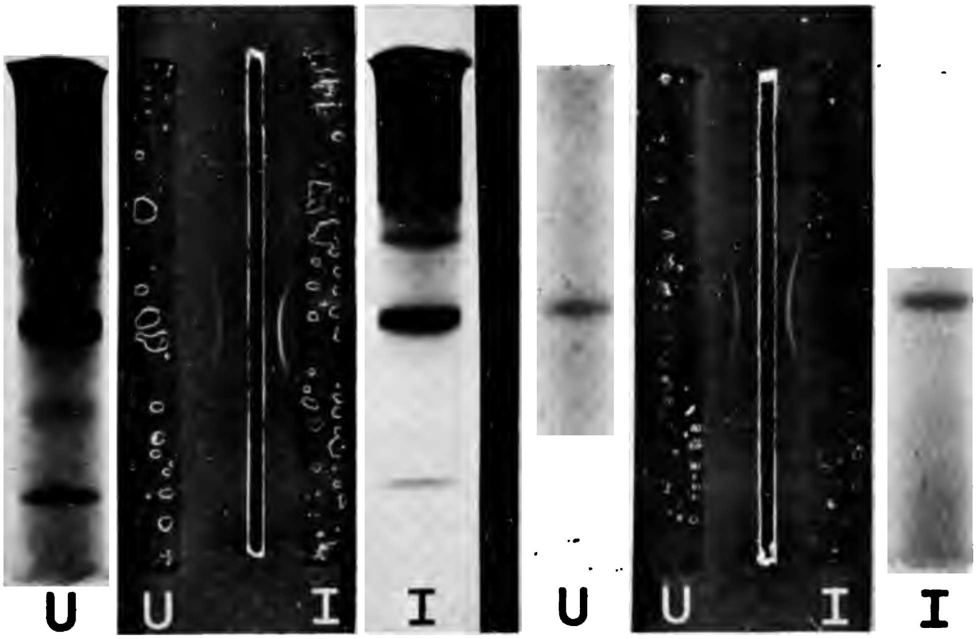

FIG. 3. Immunoelectrophoresis performed on supernatant solutions (left) and purified calcium-binding proteins (right) from hen uterus (U) and intestine (I). Amounts applied to stained gels were, as follows: uterine supernate, 200 μl; intestinal supernate, 20 μl; purified uterine calcium-binding protein, 20 μg; purified intestinal calcium-binding protein, 20 μg. Amounts applied to gels embedded for immunoelectrophoresis were: uterine supernatant, 50 μl; intestinal supernatant, 20 μl; purified uterine calcium-binding protein, 10μg; purified intestinal calcium-binding protein, 10 μg. Electrophoresis was toward the anode (bottom).

the chick intestinal and hen uterine calcium-binding proteins were electrophoretically identical.

The results of amino acid analyses performed on 22-hr acid hydrolysates of purified chick intestinal and hen uterine calcium-binding proteins are shown in Table I. Essentially no significant differences in composition exist between calcium-binding proteins isolated from these sources.

The evidence presented in this study suggests that calcium-binding proteins isolated from laying hen uterus and chick intestinal mucosa are identical with respect to electrophoretic mobility, immunological reactivity, molecular size, and amino acid composition.

Summary. Calcium-binding proteins from the chick intestine and uterus of the laying hen (*Gallus domesticus*) were isolated and purified by identical methods. Proteins from each source were shown to exhibit identical electrophoretic mobility and were immunologically identical. Estimation of molecular size by gel filtration indicated a value of approximately 28,000 daltons for both the chick intestinal and hen uterine calcium-binding proteins. The amino acid compositions of chick intestinal and hen uterine cal-

TABLE I. AMINO ACID COMPOSITION OF CHICK INTESTINAL AND HEN UTERINE CALCIUM-BINDING PROTEINS.

	Residues/molecule	
	Chick intestine	Hen uterus
Lysine	23.3	23.3
Histidine	4.3	4.3
Arginine	5.8	5.6
Aspartic acid	29.5	28.7
Threonine	8.9	8.7
Serine	9.2	9.2
Glutamic acid	34.6	35.7
Proline	2.8	3.0
Glycine	13.3	13.1
Alanine	16.6	16.6
½-Cystine	ND*	ND*
Valine	5.0	5.0
Methionine	6.9	6.1
Isoleucine	10.6	10.8
Leucine	27.6	27.2
Tyrosine	6.5	6.2
Phenylalanine	13.4	13.3

* ND is not determined.

um-binding proteins were essentially iden-
:al.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **152**, 242-245 (1976)

Protective Effect of Digitoxin in Adrenal-Compression Hypertension[1] (39370)

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Digitalis and its derivatives are the drugs of choice in the treatment of congestive heart failure, although, despite the many studies on the pharmacology of this class of compounds, their mode of action is still unclear.

Their effect on blood pressure is reported to depend on the integrity of reflexogenic areas of the cardiovascular system (1) and the presence or absence of heart disease (2). Therefore, in response to glycoside administration, blood pressure may increase (3-5), decrease (6, 7), or not change (8).

Selye (9) reported that rats treated with digitoxin (in conjunction with disodium phosphate and corn oil) develop nonocclusive myocardial necrosis. This is apparently due to increased metabolic demand, which was prevented by simultaneous administration of various substances including spironolactone, an antimineralocorticoid that is believed to promote potassium-sparing. In this regard, potassium salts or potassium-sparing agents similarly protect the rat from the toxic effects of digitalis (10).

Preliminary studies performed in this laboratory on spontaneously hypertensive (SH) rats of the Okamoto-Aoki strain indicate that cardiac glycosides may exert a hypotensive effect (unpublished observations).

This is a report on the effect of digitoxin treatment on the course of hypertension induced by adrenal compression in rats (11, 12). The cause of this type of hypertension, like that of adrenal regeneration hypertension (ARH) (13), is uncertain, but presumed to be due to an excessive secretion of one or more mineralocorticoids.

Materials and methods. Thirty female Sprague-Dawley rats weighing 75-90 g were divided into three equal groups, right-nephrectomized, and given 1% saline solution to drink and laboratory chow *ad libitum*. In addition, groups 1 and 2 underwent bilateral adrenal compression for the induction of hypertension (11). Group 3 rats which served as controls had the adrenals explored but not compressed.

From the eighth day postoperatively, the earliest time at which hypertension could be expected to occur, until the twenty-sixth day, all rats were given 1 ml of 0.9% saline per 100 g body wt daily by gavage. In the case of group 2, each milliliter contained, suspended, 0.5 mg of digitoxin powder (Parke, Davis & Co., Detroit, Mich.).

Weekly systolic blood pressure, electrocardiogram (ECG), and heart rate recordings were obtained on unanesthetized animals using a Narco-Biosystems PE-300 Programmed Electrosphygmomanometer and appropriate modules attached to a DMP-4B Physiograph (Narco-Biosystems, Inc., Houston, Texas). Pressures in excess of 150 mm Hg were regarded as indicative of hypertension.

On Day 27, the rats were sacrificed with an overdose of ether and autopsied. Various organs were removed, examined for lesions, and placed in fixative (10% neutral formalin). The organs were subsequently weighed and the weights expressed as mg per 100 g of final body weight. The lesions were graded by modified established criteria (14) as follows: 0 indicates no abnormality; 1, few small nodules, hypertrophy; 2, moderately nodular with some mottling; 3, greater nodularity, mottled with red spots; 4, rough, mottled, and flea-bitten appearance. The average severity of lesions was obtained by dividing the total score by the number of rats in the group.

The two-tailed Student's *t* test was used to determine the significance of differences between group means. Confidence limit was set at the 5% level, a *P* value of 0.05 or less indicating significance.

Results. Blood pressure. Two rats in

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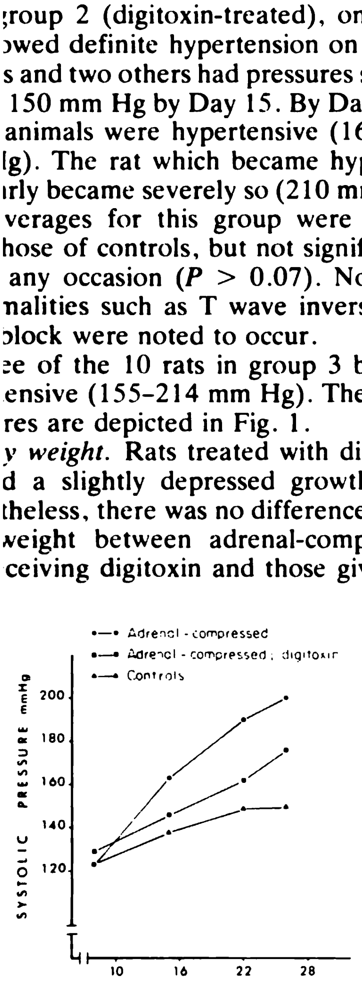
1 (compressed adrenals; not given in) died the first week; one of pneu- , the other of hydronephrosis. Data se are not reported here. In the surviystolic pressure rose rapidly so that by 5, five of eight rats had pressures g from 157-197 mm Hg. By Day 25, imals had readings in excess of 150 lg (164-230 mm Hg). The averages ignificantly different ($P < 0.03$) from ls on all occasions after the first week. roup 2 (digitoxin-treated), only one owed definite hypertension on all oc-s and two others had pressures slightly 150 mm Hg by Day 15. By Day 25, 8 animals were hypertensive (160-210 lg). The rat which became hypertenrly became severely so (210 mm Hg). verages for this group were higher hose of controls, but not significantly any occasion ($P > 0.07$). No ECG nalities such as T wave inversion or block were noted to occur.

e of the 10 rats in group 3 became ensive (155-214 mm Hg). The blood res are depicted in Fig. 1.

y weight. Rats treated with digitoxin d a slightly depressed growth rate. theless, there was no difference in the weight between adrenal-compressed ceiving digitoxin and those given saline, because the latter became ill as a result of very severe hypertension. Neither group, however, differed significantly from control (Table I).



1. Before treatment became effective (first es-), there were no differences between the group systolic pressures. Thereafter the pressures of d experimental rats were significantly higher controls on all occasions ($P < 0.03$), whereas digitoxin-treated rats occupied an intermediate nd were not significantly higher on any occa- > 0.07).

Organ weights. Untreated rats with compressed adrenals had greatly enlarged hearts, kidneys and adrenals, atrophic thymus glands, and very severe renal lesions (Table I). In contrast, although there was slight cardiac hypertrophy in the digitoxin-treated group, kidney enlargement did not occur. Furthermore, except for two of the rats which developed moderately severe hypertension and nephrosclerosis, such organs from digitoxin rats were indistinguishable from controls and neither adrenal enlargement nor thymic atrophy occurred.

Discussion. Although digitoxin treatment instituted one week following adrenal compression did not entirely prevent the development of hypertension, it did nevertheless retard the process, ameliorate its severity, and reduce lesion formation (as observed macroscopically) in the kidneys. It remains to be seen whether or not digitoxin (at 0.5 mg/ml/100 g of body weight by gavage) would be more effective in abrogating adrenal-compression hypertension if treatment were started immediately following sensitization (adrenal compression, uninephrectomy and 1% NaCl feeding) rather than deferring treatment until hypertensive changes were expected to appear.

Digitoxin would presumably also be effective in other forms of adrenal injury hypertension (enucleation, puncture) and perhaps hypertension due to steroid overdosage.

The mechanism(s) by which digitoxin modulates the hypertensive process is(are) uncertain. The glycoside may exert its effect on baroreceptors (15) causing them to respond "effectively" in counteracting pressure elevations.

Another possibility is that, because of its structural similarities, digitoxin competes with the hypertensogenic steroid for mineralocorticoid receptors thereby normalizing electrolyte balance. Selye *et al.* (16) have reported that spironolactone antagonizes digitoxin-induced arrhythmias and prevents myocardial lesion formation. Other substances as well exhibit antagonistic effects to digitoxin and other digitalis drugs. For

TABLE I. PERTINENT FINDINGS IN RATS WITH COMPRESSED ADRENALS, WITH AND WITHOUT DIGITOXIN TREATMENT.

		Group 1 Compressed adrenals	Group 2 Compressed adrenals + digitoxin	Group 3 Controls
Number of animals	Initial	10	10	10
	Final	8	10	10
Body weight (g)	Initial	84 ± 2^{a}	85 ± 1	85 ± 1
	Final	153 ± 9	153 ± 6	170 ± 7
Incidence of hypertension* (%)		100	80	30
Renal lesions	Incidence (%)	86	20	0
	Severity	2.4	0.5	0
Organ weights (mg%)	Heart	481 ± 28^{b,c}	412 ± 16^{b}	358 ± 13
	Kidney	893 ± 54^{b,c}	719 ± 33	694 ± 21
	Thymus	81 ± 32^{b,c}	154 ± 9	160 ± 14
	Adrenals	44.2 ± 4.6^{b}	36.0 ± 2.7	32.7 ± 1.4

[a] Mean ± SE.

[b] Different from control group ($P < 0.05$).

[c] Different from other experimental group ($P < 0.05$).

* Systolic pressure 150 mm Hg or greater.

instance, whereas toxic doses of ouabain (97 mg/kg, ip) will kill the animals within 40-50 min, ATP (50 mg/kg, pH 7.2) injection immediately following administration of ouabain not only protects the rat from death, but also from any cardiac rhythm disturbances (17).

In terms of blood pressure response, there are reports that glycosides cause pressure elevation in anesthetized (3, 5) as well as conscious animals (18) and in both patients free of (19) and affected by cardiovascular disease (20). On the other hand, there is an indication that they exert both a hypotensive (6, 7) and other protective effects, thus reducing morbidity and mortality from cardiac failure due to aortic constriction (2) and shock (21, 22).

Of particular significance is the finding that in addition to antagonizing cardiac (2) and renal (*vide supra*) hypertrophy, digitoxin inhibited lesion formation in these organs, in spite of the moderate hypertension observed in this experiment. It also prevented the thymus involution and adrenal enlargement, changes associated with the stress state evoked by severe hypertension.

Summary. Bilateral compression of the adrenal glands combined in mononephrectomy and followed by the imposition of a high NaCl intake resulted in severe hypertension in all rats so treated. It was accompanied by enlargement of the heart, kidneys, and adrenal glands, atrophy of the thymus, and the occurrence of severe nephrosclerosis. Digitoxin treatment delayed the onset, reduced the incidence, and ameliorated the magnitude of the hypertensive response in such animals; it also reduced the degree of cardiac hypertrophy and the severity of nephrosclerosis and completely prevented enlargement of the adrenals and kidneys and atrophy of the thymus.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 246–248 (1976)

The Inhibition by ADP of NADPH-Supported Adrenal Steroid 11β-Hydroxylation[1] (39371)

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Accumulating evidence suggests the possibility that, in the adrenal cortex, the sequence of reactions from cholesterol to corticosterone or cortisol may be functionally a highly integrated system. In addition to the primary control by the adrenocorticotropic hormone (ACTH) which takes place at the transformation of cholesterol to pregnenolone, a series of secondary controls of the steroidogenic pathway appear to be present. Thus, a number of investigations have shown a relationship between steroid 11β-hydroxylation and oxidative phosphorylation (1–5) and steroid 11β-hydroxylation can inhibit the conversion of cholesterol to pregnenolone but the reverse situation does not occur (6). Ca^{2+} has been found to stimulate both pregnenolone synthesis (7) and 11β-hydroxylase (8). Reduced NAD^+ inhibits the formation of progesterone from pregnenolone and this inhibition can be reversed by ascorbic acid and other substances (9). Both NAD^+ and NADH stimulate Δ5-3-keto-steroid isomerase (10).

It has been found recently that the succinate supported 11β-hydroxylation of deoxycorticosterone (DOC) to form corticosterone in rat adrenal mitochondria is inhibited by ADP (11). Much of the data can be explained on the basis of a competition for reducing equivalents between the hydroxylase system and the cytochrome chain. However, some observations suggested the possibility that ADP may also effect the hydroxylase system directly. In this report data is presented which indicate that ADP can indeed directly inhibit steroid 11β-hydroxylation.

Materials and methods. Mitochondria were prepared from rat adrenals as described previously (12). Following the second 0.25 *M* sucrose wash, the mitochondrial pellet was suspended in a volume of 12 m*M* Tris, pH 7.5, equal to that of the original homogenate (90 mg wet weight of tissue per ml) and contained 2.0 to 2.5 mg of protein per ml as determined by the method of Lowry *et al.* (13).

The complete system for the 11-hydroxylation of deoxycorticosterone (DOC) to form corticosterone consisted of 0.2 ml of the mitochondrial preparation, 36 m*M* Tris, pH 7.5, 20 m*M* $CaCl_2$, 30 μM DOC, 0.07 ml of NADPH prepared by a preliminary incubation for 15 min at room temperature of a solution containing, per ml, 25 μmol of $NaNADP^+$, 70 of μmole Na_2 glucose-6-P, and about 2000 units of glucose-6-P dehydrogenase, other additions as indicated and 0.25 *M* sucrose to a final volume of 1.25 ml. The DOC was added in 0.01 ml of ethanol and an incubation control with 0.01 ml of ethanol without DOC was routinely carried out. The incubations were carried out at 20° in a Dubanoff shaking incubator for 6 min, at which time 0.4-ml aliquots were delivered into 3 ml of dichloromethane and, after shaking and centrifugation, 2 ml of the dichloromethane taken for corticosterone determination by a modification (12) of the fluorometric method of Glick *et al.* (14).

Results. The incubation conditions used permit the 11β-hydroxylation of deoxycorticosterone to be supported directly by exogenous NADPH. This would obviate a competition by the hydroxylase and the cytochrome chain for reducing equivalents derived from a Krebs cycle acid such as succinate. It is seen from the data in Table I that 11β-hydroxylase supported by NADPH as the source of electrons is inhibited by ADP and ATP. ADP is more inhibitory than ATP, as was found with intact mitochondria (11) and that a clear inhibition is seen at concentrations as low as 50 μM.

Ca^{2+}, which is present in these incubations, is known to induce swelling in adrenal

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TABLE I. THE EFFECT OF ADP AND ATP ON THE FORMATION OF CORTICOSTERONE FROM DEOXYCORTICOSTERONE.

	ADP		ATP	
Nucleotide concentration (mM)	nmole corticosterone per ml	Percentage change	nmole corticosterone per ml	Percentage change
0	13.0		13.0	
0.01	13.0	0	13.4	0
0.05	9.07	−30	12.1	−7
0.10	6.34	−51	11.5	−12
0.50	2.88	−78	7.06	−46
1.0	1.58	−88	4.90	−62

mitochondria (8, 15) and ADP and ATP have been found to inhibit this swelling (15). The inhibition of the 11-hydroxylase by ADP may result from a modification of the mitochondrial membranes so that the entry of NADPH is affected. Several experiments were carried out to explore this possibility. The data in Table II show that the presence of several different types of detergents, which would be expected to disrupt mitochondrial structure, the inhibition by ADP persists. In the presence of some detergents the sensitivity of the hydroxylase to Ca^{2+} changes and Ca^{2+} concentration has been adjusted to obtain maximal activity.

In the experiments presented in Table III the effects on the ADP inhibition of preincubation of the mitochondria under various conditions has been determined. It is seen that the ADP inhibition is still present after a 10-min preincubation in the presence of Ca^{2+}, NADPH, and ADP or in the presence of Ca^{2+} and NADPH. There is a small decrease in the inhibition under the latter preincubation condition.

TABLE II. THE INHIBITION OF 11-HYDROXYLATION BY ADP IN THE PRESENCE OF VARIOUS DETERGENTS.[a]

	ADP		
Addition	nmole corticosterone/ml		Percentage change
Lubrol	18.0	1.8	−90
Triton X-100	13.8	1.5	−89
Deoxycholate	18.1	5.8	−68

[a] ADP, when present, was 2 mM. The concentrations of the detergents, per milliliter of incubation medium, were Lubrol, 40 μg; Triton X-100, 80 μg; Na deoxycholate, 80 μM. Ca^{2+} concentrations in incubations with Lubrol was 20 mM, and in those with Triton X-100 and deoxycholate were 8 mM.

TABLE III. THE EFFECT OF PREINCUBATION IN THE ABSENCE AND PRESENCE OF ADP ON THE INHIBITION OF 11-Hydroxylase by ADP.[a]

	ADP		
Preincubation	nmole corticosterone/ml		Percentage change
A	17.2	1.5	−91
B	22.2	5.2	−77
C	21.3	2.3	−89

[a] Preincubation: A, none. B, 10 min with the complete system except for DOC. ADP, when present, was added in the final incubation. The reaction was started with DOC. C, the preincubation was the same as in B except that ADP, when added, was present during the preincubation. The reaction was started with DOC. ADP, when present, was 2 mM.

Discussion. A number of observations indicates that the ADP effect is due to an action directly upon the hydroxylase system. The ADP inhibition persists in the presence of various detergents as well as when the mitochondria are preincubated with Ca^{2+} and NADPH where the possibility of an ADP induced impairment of NADPH entry into the mitochondrial membranes is not present. In support of this conclusion is the observation that while ADP is more inhibitory than ATP, ATP is more effective than ADP in preventing the Ca^{2+} induced swelling of adrenal mitochondria (15). The ADP inhibition of 11β-hydroxylase activity is somewhat less when the mitochondria are preincubated with Ca^{2+} and NADPH (77%) than when ADP is also present during this preincubation (89%). This may indicate a possible minor effect of ADP upon NADPH entry into the mitochondrial membranes. Further work will be required to clarify the mechanism of the ADP inhibition as well as the Ca^{2+} stimulation of this enzyme system.

Summary. The 11β-hydroxylation of deoxycorticosterone to form corticosterone in adrenal mitochondria has been found to be inhibited by ADP and ATP, with ADP being the more inhibitory of the two. The evidence suggests that the ADP directly affects the enzyme system.

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Inhibitory Effect of Vitamin E on Desferrioxamine-Induced Iron Excretion in Rats[1] (39372)

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n overload is a major limiting factor in fe expectancy of patients with severe s of congenital hemolytic anemia such alassemia major, particularly in those nts who are participating in hypertrans- n programs. Treatment with iron che- ; agents has been introduced in an at- t to reduce excessive iron accumulation ese patients. The most commonly used chelating drug is desferrioxamine (DF), 1 has been shown to interact mainly parenchymal iron deposits with subse- t excretion in urine and bile (1). Perox- on of membrane lipids and proteins was ibed in thalassemic erythrocytes in as- tion with markedly reduced serum lev- alpha tocopherol (vitamin E) a known xidant (2). Preliminary studies have n that some parameters reflecting brane peroxidation can be corrected by dministration of large doses of vitamin). It is probable that both antioxidants ron chelating drugs will be employed taneously in the future management of ssemic patients. The present study was rtaken in order to evaluate the effect of iin E on the mobilization and excretion on following DF administration to nor- ind iron overloaded rats.

thods. Female Wistar rats (Hadassah ı) weighing 170 to 200 g were used ghout. Injections were performed gh the tail veins. Animals were sacri- under ether anesthesia by exsanguina- into heparinized syringes through the minal aorta. Parenteral iron overload nduced by four consecutive injections of 12 mg of iron dextran within 2 weeks. All procedures involving iron analysis were carried out in iron-free glassware. Ferritin was isolated from tissue homogenates by the method of Fulton and Ramsay (4) employing ammonium sulfate, incubated overnight in 3 *N* HCl at 37° and its iron content was measured colorimetrically. Total non-heme iron was determined by the method of Torrance and Bothwell (5). Serum vitamin E levels were determined by the method of Hashim and Schuttringer (6).

Counting techniques. The radioactivity of spleen, weighed portions of the liver, and 1-ml samples of blood were determined in an automatic well-type scintillation counter (Packard model 3003 spectrometer). Whole body counts were performed in a small animal counter (Packard model 447, Packard Instruments Co., Downers Grove, Illinois). Radioiron excretion was determined by whole body counts on the first and last days of the study, with corrections for decay and differences in geometry. Hepatic radioactivity was determined by relating sample counts to total organ weight. Residual radioactivity was defined as whole body radioactivity minus the combined activities of blood, liver, and spleen (1).

Preparation and initial processing of ^{59}Fe-ferritin. In vivo-labeled ferritin was prepared by injecting 200 μCi citrated ^{59}Fe into iron overloaded rats. The animals were sacrificed 24 hr later, and purified radioactive ferritin was prepared by the method of Bjorklid and Helgeland (7). Acrylamide gel electrophoresis of the ferritin preparation at pH 8.5 produced a single protein band, and a single precipitin line was demonstrated on immunodiffusion against antiferritin serum. The specific activity of radioferritin was 4 μCi per mg iron. The initial processing of soluble ferritin ^{59}Fe has been described in

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detail in a previous communication (1). Briefly, 89 ± 1% of the injected dose were located to the liver within 1 hr. The cellular distribution of radioactivity in the liver, determined by a quantitative autoradiographic method (8) was 0 to 3% in R.E. cells and 97 to 100% in hepatic parenchymal cells. The half time disappearance of intravenously injected soluble ferritin was 16 ± 1 min. Within 1 day of injection about 80% of the hepatic radioactivity could be recovered in the ferritin fraction of tissue homogenates, and the rest in soluble nonferritin iron.

Outline of study. Three weeks prior to labeling, 40 rats were divided into two equal groups of normal and iron-overloaded animals. In addition, half of each group was given 50 i.u. of dl-alpha-tocopherol (Hoffman La Roche, Nutley, New Jersey) by gastric tube daily for 3 weeks prior to radioiron labeling, and continued throughout the study. On the first day of study all animals were labeled by iv injection of ^{59}Fe ferritin at a dose representing 40 μg of iron per rat. Thereafter, one-half of each group was treated by injections of 10 mg of DF (Desferral, Ciba Pharmaceutical Co., Summit, N.J.) twice daily for 10 consecutive days.

Results. The mean serum vitamin E level of the normal and iron overloaded groups of rats was 0.68 ± 0.15 and 0.95 ± 0.15 mg/dl (± SD), respectively. Following vitamin E administration there was a twofold increase in serum vitamin E levels in both groups to 1.43 ± 0.29 and 1.75 ± 0.21 mg/dl, respectively. The effect of vitamin E on iron chelation in normal, and iron overloaded animals was evaluated by the measurement of total hepatic nonheme iron and ferritin iron stores (Table I). In normal controls 62 ± 4% of the total hepatic iron was found in ferritin. DF administration resulted in a significant reduction of ferritin iron in both control and vitamin E-treated groups, and a similar, but less pronounced decrease in total nonheme iron. Unlike normal animals in iron overloaded rats, DF-induced reduction in ferritin iron and total nonheme iron was completely inhibited following vitamin E administration.

From the above results, one could not distinguish between a direct effect of vitamin E and DF on hepatic iron stores, or an extracellular effect manifested indirectly in altered rates of storage iron accumulation. The former possibility was examined by employing ^{59}Fe-ferritin as a selective parenchymal radioiron probe. As shown in Table II, DF-induced radioiron excretion was restricted to hepatic parenchyma. The reduction in hepatic Fe^{59} activity induced by DF administration was almost equal to the enhancement of whole body ^{59}Fe excretion found in the same animals. No significant changes in radioactivity could be observed in any of the other organs examined. Vitamin E administration did not alter radioiron excretion following DF treatment in normal rats. However, in iron overloaded rats the effect of DF was completely abolished when vitamin E was given.

The possibility of a direct effect of vitamin E on ferritin iron chelation was studied *in vitro* by incubating aliquots of ^{59}Fe-ferritin representing 50 μg of iron with 1 mmole of DF with or without the addition of 10 u of vitamin E, followed by overnight dialysis in Tris–HCl buffer 0.175 *M* at pH 8.4. Only 0.2 ± 0.1% of the radioactivity were removed by dialysis from the control sample

TABLE I. Total Hepatic Non-Heme Iron and Ferritin Iron.[a]

Treatment	Normal		Iron Overload	
	Nonheme	Ferritin	Nonheme	Ferritin
Control	1626 ± 106†	1009 ± 98	15,110 ± 658	4872 ± 206
Control + DF	1311 ± 40*	560 ± 52***	10,602 ± 1277**	3600 ± 373**
Vitamin E	1588 ± 111	733 ± 72	13,926 ± 1206	4393 ± 192
Vitamin E + DF	1313 ± 120 n.s.	551 ± 46*	15,425 ± 1066 n.s.	4540 ± 202 n.s.

[a] Micrograms of Fe per organ.

BLE II. ORGAN DISTRIBUTION OF RADIOACTIVITY AT 10d (PERCENTAGE OF INJECTED ^{59}Fe-FERRITIN).

tion	Treatment	Blood	Liver	Spleen	Residual	Excretion
	Control	$14.5 \pm 1.6^{\dagger}$	76.5 ± 2.5	0.7 ± 0.1	2.3 ± 0.2	6.0 ± 0.9
	Control + DF	13.7 ± 0.7	$56.9 \pm 1.3^{\bullet\bullet\bullet}$	0.8 ± 0.1	1.6 ± 1.2	$27.0 \pm 0.7^{\bullet\bullet\bullet}$
	Vitamin E	15.3 ± 1.2	72.9 ± 4.0	0.7 ± 0.1	5.4 ± 3.8	5.7 ± 1.3
	Vitamin E + DF	15.4 ± 2.6	$54.2 \pm 3.5^{\bullet\bullet\bullet}$	0.7 ± 0.1	2.0 ± 2.4	$27.7 \pm 0.8^{\bullet\bullet\bullet}$
ɔad						
	Control	3.8 ± 0.5	75.9 ± 2.4	0.8 ± 0.1	12.1 ± 2.3	7.4 ± 1.9
	Control + DF	4.8 ± 0.5	$59.9 \pm 1.9^{\bullet\bullet\bullet}$	0.9 ± 0.1	18.2 ± 3.1	$16.2 \pm 1.4^{\bullet\bullet\bullet}$
	Vitamin E	4.1 ± 0.2	57.6 ± 8.8	1.3 ± 0.6	20.0 ± 9.5	7.0 ± 0.6
	Vitamin E + DF	5.2 ± 0.6	69.4 ± 4.1 n.s.	0.9 ± 0.1	15.5 ± 5.8	9.0 ± 2.3 n.s.

0.05.
: 0.01.
< 0.001.
n ± 1 SE.

ɪt DF, as compared to 27.7 ± 0.1%
ɔF and 28.9 + 0.9% with DF and
n E.

:ussion. The mode of action of the
helating agent DF has been studied
ɪively in man and in experimental ani-
9). Using selective radioiron probes of
ɪs iron compartments, it was shown
ɔF interacts primarily with hepatocel-
ferritin (1). Chelated iron is subse-
ly eliminated through biliary and uri-
xcretion. DF-induced iron excretion is
/ related to the magnitude of iron
, and its measurement is widely used
agnostic tool in the assessment of iron
ɪad (9). However, factors other than
ze of iron stores might affect the
ɪts of iron removed by DF. Depletion
ɪmin C, often associated with iron ov-
, was shown to reduce response to
:onversely, vitamin C administration
; in a marked enhancement of DF-
:d iron excretion (10). Very low se-
itamin E levels were found in patients
ɜ-thalassemia major with varying de-
of iron overload (2). Thus, it was
ated that in analogy with vitamin C,
lministration of vitamin E might also
y iron chelation by DF.

he present study, DF-induced iron ex-
ɪ was completely inhibited when vi-
E was administered in pharmacologi-
ses to rats with iron overload. Theo-
ly, this inhibition could be the result
ɪer (a) direct interference with ferri-
interaction; (b) increased break-
ɔf DF by microsomal drug-metaboliz-
ɪtems, or (c) prevention of membrane
ɪeroxidation in iron overloaded ani-
mals, resulting in reduced permeability to either DF or the DF–iron complex. The first possibility is least likely since in the present study, the addition of vitamin E did not inhibit *in vitro* ^{59}Fe-ferritin chelation by DF. Experimental evidence in support of vitamin E action on drug metabolism and membrane protection is available (11, 12), but to what extent this is related to the present findings is as yet unknown. The inability to demonstrate a similar inhibitory effect of vitamin E on DF-induced iron chelation in normal rats is probably explained by their smaller ferritin pool, for which partial inhibition of a relative excess of DF may not be a limiting factor.

Long-term clinical studies employing vitamin E and desferrioxamine in the management of thalassemic patients with transfusion-induced iron overload are ongoing in several centers. The demonstration of an inhibitory effect of vitamin E on DF-induced iron excretion may have significant practical implications in the planning of therapy for thalassemic patients with severe secondary iron overload.

Summary. The influence of vitamin E on the mobilization and excretion of storage iron induced by DF was studied in normal and iron-overloaded rats. Vitamin E administration in pharmacologic doses resulted in complete inhibition of the effect of DF on storage iron in iron-overloaded rats while no such effect could be demonstrated in rats with normal iron stores. The mechanism of the observed inhibition of DF action by vitamin E is at present unknown. Nevertheless this drug interaction has to be considered in view of ongoing therapeutic trials

where both antioxidants and iron chelating drugs are administered simultaneously to thalassemic patients with transfusion induced iron overload.

The skillful technical assistance of Ludmilla Eilon, Luda Gelfand, and Aliza Shifter is gratefully acknowledged.

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NGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 253-256 (1976)

ective Effects of Cholestyramine in Rats Fed a Low-Fiber Diet Containing Toxic Doses of Sodium Cyclamate or Amaranth (39373)

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ailable data indicate that a number of
additives are toxic when fed in con-
ion with a purified, low-fiber diet at
;es which have little, if any, deleterious
: when fed with diets containing dietary
(1-6). However, marked differences
in the protective effect of different die-
fibers in this regard, with blond psyl-
seed powder being a particularly po-
ource of an active factor or factors (6).
: present communication, data are pre-
d indicating that the anion exchange
cholestyramine has activity similar to
of blond psyllium seed powder in coun-
:ing the toxic effects of massive doses
dium cyclamate and amaranth (FD&C
No. 2) in rats fed a purified, low-fiber

ocedure and results. The basal purified,
iber diet employed in the present study
sted of sucrose, 66%; casein,[1] 24%;
nixture,[2] 5%; cottonseed oil, 5%; and
ollowing vitamins per kilogram of diet:
ine hydrochloride, 5 mg; riboflavin, 5
pyridoxine hydrochloride, 5 mg; cal-
pantothenate, 50 mg; nicotinic acid,
ng; ascorbic acid, 200 mg; para-ami-
nzoic acid, 200 mg; inositol, 400 mg;
., 1 mg; folic acid, 5 mg; vitamin B_{12},
μg; 2-methyl, 1-4 naphthoquinone, 5
holine chloride, 2 g; vitamin A, 5,000
units; vitamin D_2, 500 USP units; and
opheryl acetate, 100 mg. The vitamins
added in place of an equal amount of
se. In the initial experiment 48 male
f the Sprague-Dawley strain were se-
l at an average body weight of 50.2 g
:, 44 to 58 g) and were divided into 8
arable groups of 6 animals each. One
was fed the basal purified, low-fiber
ndicated above; a second group was fed the basal purified, low-fiber diet + 5% sodium cyclamate;[3] the remaining groups were fed the basal purified, low-fiber diet + 5% cyclamate + cholestyramine[4] or blond psyllium seed powder at the levels indicated in Table I. Sodium cyclamate, cholestyramine, and blond psyllium seed powder were incorporated in the diets in place of an equal amount of sucrose. Animals were placed in metal cages with raised screen bottoms (three rats per cage) and were provided the test diets and water *ad libitum*. Animals were fed daily and all food not consumed 24 hr after feeding was discarded. Body weight was recorded weekly. Feeding was continued for 14 days or until death, whichever occurred sooner.

In agreement with previous findings (3, 6), the addition of sodium cyclamate at a 5% level to the basal purified, low-fiber diet resulted in a highly significant retardation in weight increment, lack of grooming, varying degrees of alopecia and extensive diarrhea with watery and mushy stools. Of six rats fed the latter diet, five succumbed during the experimental period of 14 days. Blond psyllium seed powder when incorporated in the latter diet had a marked protective effect which was proportional to the level fed. The protective effect of blond psyllium seed powder was particularly marked at the 5% level of feeding. In rats fed the latter diet the fur appeared smooth and sleek and with the exception of a mild diarrhea and soft but well-formed stools the animals appeared normal in gross appearance. Cholestyramine when fed with the basal purified, low-

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itamin-Free Test Casein, Teklad Test Diets,
on, Wisconsin.

ineral Mix, U.S.P. XVII. Teklad Test Diets.
on, Wisconsin.

[3] The sodium cyclamate employed in the present experiment was kindly provided by Dr. Ronald G. Wiegand of Abbott Laboratories, North Chicago, Illinois.

[4] The cholestyramine employed in the present study (anhydrous cholestyramine) was kindly provided by Dr. Herbert P. Sarett of the Mead Johnson Research Center, Evansville, Indiana.

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TABLE I. COMPARATIVE EFFECTS OF GRADED LEVELS OF BLOND PSYLLIUM SEED POWDER AND CHOLESTYRAMINE ON THE WEIGHT INCREMENT OF IMMATURE MALE RATS FED A PURIFIED, LOW-FIBER DIET + 5% SODIUM CYCLAMATE (SIX ANIMALS PER GROUP).[a]

Dietary group	Average gain (g) in body weight after following days of feeding: 7 days	14 days
Basal purified, low-fiber diet	34.3 ± 5.4[b]	79.8 ± 8.4[b]
Purified, low-fiber diet + 5% sodium cyclamate	5.8 ± 5.8 (4)	19.0 ± 0.0 (1)
Purified, low-fiber diet + 5% sodium cyclamate + following supplements:		
1¼% blond psyllium seed powder	6.6 ± 7.1	24.1 ± 10.4
2½% blond psyllium seed powder	17.2 ± 6.6	51.3 ± 5.3
5% blond psyllium seed powder	31.6 ± 4.8	77.2 ± 7.4
1¼% cholestyramine	12.2 ± 5.6	38.2 ± 9.2
2½% cholestyramine	31.0 ± 4.7	79.2 ± 7.5
5% cholestyramine	29.3 ± 7.4	75.4 ± 12.8

[a] The average initial body weight of rats in the various groups was 50.2 g. The values in parentheses indicate the number of animals which survived and on which data are based when this number was less than the original number per group.

[b] Standard error of the mean.

fiber + 5% sodium cyclamate diet also had a marked protective effect. At both the 1¼ and 2½% levels of feeding, cholestyramine promoted an increment in body weight which was in excess of 50% greater than that of rats fed an equal amount of blond psyllium seed powder. Rats fed the diet containing 2½% cholestyramine were indistinguishable grossly from those fed the ration containing 5% blond psyllium seed powder. No deaths occurred on any of the diets containing either the blond psyllium seed powder or cholestyramine supplements. The effects of the various diets on the number of animals surviving and the average increment in body weight are indicated in Table I.

Tests were next conducted on the comparative effects of cholestyramine and other anion exchange resins as well as cation exchange resins in counteracting the toxic effects induced by sodium cyclamate when incorporated at a 5% level in the basal purified, low-fiber diet. Sixty-six male rats of the Sprague–Dawley strain were selected at an average body weight of 47.7 g (range 45 to 50 g) and were divided into 11 comparable groups of six animals each. One group was fed the basal purified, low-fiber diet; a second group was fed the basal purified, low-fiber diet + 5% sodium cyclamate; the remaining groups were fed the latter diet + either cholestyramine or a strongly basic anion exchange resin,[5] a weakly basic anion exchange resin,[6] a strongly acidic cation exchange resin,[7] or a weakly acidic cation exchange resin[8] at the levels indicated in Table II. Sodium cyclamate and the anion and cation exchange resins were incorporated in the diets in place of an equal amount of sucrose. The experimental procedure was similar to that employed in the initial experiment.

In agreement with findings in the previous experiment, the addition of sodium cyclamate at a 5% level to the basal purified, low-fiber diet resulted in toxic manifestations similar to those described above, effects that were counteracted by the concurrent administration of cholestyramine at a 2½% level in the diet. A marked protective effect also occurred in rats fed the strongly basic anion exchange resin, particularly when the latter was fed at a 5% level in the diet.[9] A moderate protective effect was observed in rats fed the weakly basic anion exchange resin. Both the strongly acidic and

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[5] Amberlite Ion Exchange Resin No. 3335 (Rohm & Haas No. IRA-400); moisture content, 42 to 48%; Mallinckrodt Chemical Works, St. Louis, Missouri.

[6] Amberlite Ion Exchange Resin No. 3328 (Rohm & Haas No. IR-45); moisture content, 45%; Mallinckrodt Chemical Works, St. Louis, Missouri.

[7] Amberlite Ion Exchange Resin No. 3323 (Rohm & Haas No. IR-120); moisture content, 44 to 48%; Mallinckrodt Chemical Works, St. Louis, Missouri.

[8] Amberlite Ion Exchange Resin No. 3324 (Rohm & Haas No. IRC-50); moisture content, 43 to 53%; Mallinckrodt Chemical Works, St. Louis, Missouri.

[9] Since the moisture content of this product was approximately 50% (see footnote 5), a 5% supplement of this material was comparable on a dry weight basis to a 2½% supplement of cholestyramine.

TABLE II. COMPARATIVE EFFECTS OF CHOLESTYRAMINE, OTHER ANION EXCHANGE RESINS AND CATION EXCHANGE RESINS ON THE WEIGHT INCREMENT OF IMMATURE MALE RATS FED A PURIFIED, LOW-FIBER DIET + 5% SODIUM CYCLAMATE (SIX ANIMALS PER GROUP).[a]

Dietary group	Average gain (g) in body weight after following days of feeding:	
	7 days	14 days
Basal purified, low-fiber diet	37.5 ± 3.2[b]	83.0 ± 7.1[b]
Purified, low-fiber diet + 5% sodium cyclamate	11.0 ± 2.7 (3)	26.5 ± 7.8 (2)
Purified, low-fiber diet + 5% sodium cyclamate + following supplements:		
2½% cholestyramine	33.8 ± 5.2	81.2 ± 7.2
2½% Amberlite Ion Exchange Resin No. 3335, a strongly basic anion exchange resin	17.5 ± 4.1	53.6 ± 7.4
5% Amberlite Ion Exchange Resin No. 3335	30.3 ± 4.6	71.0 ± 3.6
2½% Amberlite Ion Exchange Resin No. 3328, a weakly basic anion exchange resin	10.6 ± 4.4	38.2 ± 5.9
5% Amberlite Ion Exchange Resin No. 3328	13.3 ± 5.3	52.5 ± 7.7
2½% Amberlite Ion Exchange Resin No. 3323, a strongly acidic cation exchange resin	8.0 ± 4.6 (3)	27.0 ± 7.2 (3)
5% Amberlite Ion Exchange Resin No. 3323	5.2 ± 1.7 (5)	28.5 ± 1.0 (2)
2½% Amberlite Ion Exchange Resin No. 3324, a weakly acidic cation exchange resin	8.3 ± 2.6 (4)	34.5 ± 2.2
5% Amberlite Ion Exchange Resin No. 3324	−3.0 ± 0.0 (1)	

[a] The average initial body weight of rats in the various groups was 47.7 g. The values in parentheses indicate the number of animals which survived and on which data are based when this number was less than the original number per group.

[b] Standard error of the mean.

weakly acidic cation exchange resins were without protective effect. The effects of the various diets on the number of animals surviving and the average increment in body weight are indicated in Table II.

Tests were subsequently conducted on the comparative effects of cholestyramine and blond psyllium seed powder in counteracting the toxic effects induced by amaranth when incorporated at a 5% level in the basal purified, low-fiber diet. Thirty male rats of the Sprague–Dawley strain were selected at an average body weight of 49.2 g (range 46 to 52 g) and were divided into five comparable groups of six animals each. One group was fed the basal purified, low-fiber diet; a second group was fed the basal purified, low-fiber diet + 5% amaranth;[10] the remaining groups were fed the latter diet + either cholestyramine or blond psyllium seed powder at the levels indicated in Table III. The experimental procedure was similar to that employed above.

In agreement with previous findings (4), amaranth when incorporated at a 5% level in the purified, low-fiber diet resulted in a virtual cessation of growth, an unthrifty appearance of the fur (which was matted and infiltrated with amaranth) and death of all animals in this group within the experimental period of 14 days. In contrast to the above, the deleterious effects indicated above were largely counteracted by the concurrent administration of either cholestyramine or blond psyllium seed powder at a 2½% level in the diet. The fur of rats fed the latter supplements was not matted nor infiltrated with amaranth although it still had an unthrifty appearance and a pink color resulting from contact with the dye in the diet; otherwise with the exception of a small retardation in weight increment these rats were indistinguishable in gross appearance from those fed the basal purified, low-fiber diet alone. Gain in body weight was slightly greater in rats fed blond psyllium seed powder at a 5% level in the diet than at a 2½% level; otherwise these rats were similar in appearance. All rats fed either the cholestyramine or blond psyllium seed-containing diets survived. The effects of the various

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[10] F.D. & C. Red No. 2 Certified (Amaranth), 94% pure dye, H. Kohnstamm & Co., New York, N.Y.

TABLE III. Comparative Effects of Cholestyramine and Blond Psyllium Seed Powder on the Weight Increment of Immature Male Rats Fed a Purified, Low-Fiber Diet + 5% Amaranth (Six Animals per Group).[a]

Dietary group	Average gain (g) in body weight after following days of feeding:	
	7 days	14 days
Basal purified, low-fiber diet	35.2 ± 4.8[b]	79.2 ± 7.5[b]
Purified, low-fiber diet + 5% amaranth	9.0 ± 0.0 (1)	
Purified, low-fiber diet + 5% amaranth + following supplements:		
2½% cholestyramine	25.5 ± 4.7	63.7 ± 7.5
2½% blond psyllium seed powder	24.7 ± 6.6	60.8 ± 5.3
5% blond psyllium seed powder	33.7 ± 5.0	72.0 ± 10.2

[a] The average initial body weight of rats in the various groups was 49.2 g. The values in parentheses indicate the number of animals which survived and on which data are based when this number was less than the original number per group.

[b] Standard error of the mean.

diets on the number of animals surviving and the average increment in body weight are indicated in Table III.

Discussion. Present findings indicate that the toxic effects observed grossly in immature male rats fed sodium cyclamate at a 5% level in a purified, low-fiber diet were counteracted by the concurrent administration of the anion exchange resin cholestyramine at a 2½% level in the diet. Other anion exchange resins were also protective in this regard with activity more marked with a strongly basic anion exchange resin than a weakly basic one. Cholestyramine at a 2½% level in the diet was also active in counteracting the toxic effects obtained in immature male rats fed a purified, low-fiber diet containing 5% amaranth.[11] Inasmuch as blond psyllium seed powder and other dietary fiber-containing materials have also been shown to be active in counteracting the toxic effects obtained in rats fed a purified, low-fiber diet supplemented with sodium cyclamate or amaranth at a 5% level (4, 6), present findings suggest that the protective effect of dietary fibers from the above sources may have been due to their anion exchange activity. The possibility that the activity of cholestyramine under conditions of the present experiment was due at least in part to some property other than its anion exchange activity, however, has not been excluded.

Summary. Immature male rats were fed a purified, low-fiber diet containing massive doses of sodium cyclamate or amaranth (FD&C Red No. 2). Sodium cyclamate when incorporated at a 5% level in the purified, low-fiber diet resulted in toxic manifestations which were counteracted by the concurrent administration of the anion exchange resin cholestyramine at a 2½% level in the diet. Other anion exchange resins were also active in this regard. Cholestyramine at a 2½% level of supplementation was also active in counteracting the toxic effects induced by amaranth when the latter was incorporated at a 5% level in the purified, low-fiber diet.

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[11] Subsequent studies conducted in this laboratory (unpublished data) indicate that cholestyramine at a 2½% level in the diet was also active in counteracting the toxic effects induced by FD&C Yellow No. 5 (tartrazine) when the latter was incorporated at a 5% level in a purified, low-fiber diet.

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ory Effect of Synthetic Phospholipid Vesicles Containing Cholesterol on the Fertilizing Ability of Rabbit Spermatozoa (39374)

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stigations undertaken in this labora- -3) indicate that two distinct classes nbrane vesicles are responsible for le inhibition of fertilizing ability in permatozoa by seminal plasma from nd vasectomized bucks (4, 5). Fertil- *in vitro* of eggs from superovulated re rats by cauda epididymal sperm as also inhibited when vesicles iso- rom rabbit seminal plasma were o the medium (6). These findings are nt with a direct, nonspecific action inal plasma vesicles on the sperm

e present investigation, the effect of composed of dipalmitoyl phosphati- ine (DPPC), dimyristoyl phosphati- ine (DMPC), and cholesterol have amined on the fertilizing ability of capacitated rabbit spermatozoa.

rials and methods. Lipid suspen- ive lipids were employed in these ents: cholesterol (Steraloids), *β-γ*- toyl-DL-*α*-glycerol-phosphorylcho- gma), *β-γ*-dimyristoyl-L-*α*-glycerol- rylcholine (Sigma), phosphatidyl-L- Nutritional Biochemical), and L-*α*- -phosphorylcholine (Sigma). Purity preparations was established, prior by thin-layer chromatography on sil- lates that were developed first with rm, methanol, and water (65:25:4) n petroleum ether, ethyl ether, and cid (80:20:1) (7). Mixtures of phos- l and cholesterol were prepared by ion in benzene, or chloroform, and ent lyophilization to remove the or- lvent. The lipids were suspended /ml) in 1 ml of Hanks solution , modified to contain 3.8 m*M* Ca^{2+} d ultrasonic irradiation was per- with a titanium probe (0.4-cm diam- tted to a Biosonik III sonicator ill) that was operated at 20 kHz. sonication, which lasted about 15 resterilized 5-ml plastic tube (Falcon) containing the lipid suspension was immersed in an ice bath. Vesicle formation was established by examination of a sample placed on a carbon-coated formvar–copper grid (200 mesh) under an electron microscope (Zeiss, EM 9S2), after negative staining with 10 mg/ml of uranyl acetate.

Assay of sperm fertilizing capacity. Treatment effects on the fertilizing capacity of rabbit spermatozoa were determined from the fertilization rates achieved among eggs obtained from does following insemination with treated and untreated sperm cells into contralateral oviducts, as previously described (3). Spermatozoa flushed from the uterus of a donor in 4 to 6 ml of Ca^{2+}-enriched Hanks solution, about 12 hr after mating, were subdivided into two equal portions, before sedimentation at 600 *g* for 15 min. Both cell pellets were then resuspended in the same volume (0.3 to 1.0 ml) of medium. Experimental (Hanks + 0.5 to 10 mg of lipid/ml) and control (Hanks) sperm suspensions, containing 0.2 to 20 × 10^6 sperm cells/ml, were preincubated for 45 min at 37° before insemination. An iv injection of 70 to 90 USP units human chorionic gonadotrophin (Squibb) induced ovulation in these animals. To assay for decapacitation activity, 10- or 25-μl control and test sperm suspensions were instilled into opposite oviducts of a doe, which had been anesthetized with 30 mg of sodium pentobarbital/kg body weight, 2 to 4 hr after the anticipated time of ovulation. The female rabbits used in these experiments were of the New Zealand strain, of proven fertility, and 3.5 to 5.5 kg body weight. The recovery of fertilizing capacity in treated spermatozoa was determined from fertilization rates observed following intrauterine injection of 0.1-ml sperm suspension with a 1-ml disposable syringe having an 0.5-in. 26-gauge needle. Sperm cell concentrations were determined with a hemocytometer. The sperm suspensions used in this investigation

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served.

showed motility at the completion of each experiment, which usually involved insemination of five does. The inseminated recipients were autopsied after 1 day and eggs flushed from their oviducts were mounted on a glass slide, stained with 10 mg/ml of lacmoid, and inspected microscopically for chromatin, polar body formation, and associated spermatozoa. There were spermatozoa in contact with the eggs recovered from all does included in the results. Differences between control and experimental fertilization rates have been assessed for statistical significance with a χ^2 test, corrected for data discontinuity.

Results. In Fig. 1 are shown vesicles prepared from DPPC and DPPC with 10 (w/w)% cholesterol. The vesicles range in apparent diameter from approximately 500 to 3000 Å in each preparation.

Data presented in Table I reveal that uterine-capacitated rabbit spermatozoa fertilized significantly fewer eggs when suspended in medium with DMPC and DPPC vesicles with 10 to 40 (w/w)% cholesterol, at concentrations of 1 to 10 mg of lipid/ml, following insemination 2 to 4 hr postovulation. Among a total of 79 eggs from oviducts of does inseminated with sperm cells exposed to these vesicles there were only 8 fertilized eggs, whereas 67 of 87 eggs from the contralateral oviduct had been fertilized by untreated spermatozoa. Fewer eggs were fertilized by sperm treated with cholesterol containing vesicles prepared with DPPC than DMPC. The DMPC-cholesterol vesicle suspensions were more inhibitory at concentrations of 9 to 10 mg of lipid/ml than at 1 mg of lipid/ml, as might be expected. In three experimental groups concerning DMPC and DPPC vesicles with cholesterol shown in Table I, the mean number of sperm cells per egg for each group ranged from 2.7 to 15.4, while control groups averaged between 7.7 and 83.8 sperm per egg. The number of spermatozoa reaching the egg does not appear to be the main cause in preventing fertilization, however, for these ranges in the mean number of sperm per egg can be seen to overlap. It was noticed that spermatozoa treated with these vesicles became less motile. It was also noted that 9.4 mg of lipid/ml of DMPC vesicles with 40 (w/w)% cholesterol, prepared from mixtures that did not involve dissolution by an organic solvent, caused a decrease in fertilization rate from 82% (18/22) with untreated spermatozoa to 5% (1/19) by treated sperm cells. In one experiment, 10 (w/w)% phosphatidylserine was added to DMPC-cholesterol vesicles, with no apparent influence on decapacitation activity. Parenthetically, there was an average fertilization rate of 84% after oviductal insemination of untreated spermatozoa and, it may be noted, an inverse relationship, albeit a weak one (regression coefficient = 0.697), exists between fertilization rate and the number of sperm inseminated, over the range (2.5 to 50.0 × 10^4 sperm per oviduct) used in these experiments.

Intrauterine insemination of uterine-capacitated spermatozoa treated with DMPC and DPPC vesicles containing 40 (w/w)% cholesterol at a concentration of 1 mg of lipid/ml, 5 to 6 hr prior to ovulation, resulted in 53% (18/34) fertilization (Table I). By comparison, 55% (23/42) eggs were fertilized with untreated spermatozoa on the control side. This suggests that the inhibitory effect of these synthetic vesicles on sperm fertilizing capacity is reversed after treated sperm cells have been returned to the uterus for recapacitation. The possibility that these spermatozoa may not have been initially in a decapacitated state can be ruled out since insemination of these sperm into the uteri of two does, about 4 hr postovulation, resulted in 8% (1/12) fertilization with DPPC-cholesterol-treated sperm cells and 92% (11/12) fertilization with untreated spermatozoa, which is a statistically significant difference ($\chi_1^2 = 13.25$, $0.05 \gg P$).

Vesicles composed of DMPC and DPPC, without cholesterol, displayed no apparent decapacitation activity, at concentrations of 1 and 10 mg of lipid/ml, respectively. Table I shows that among both groups 80% (28/35) eggs were fertilized by sperm cells treated with phosphatidylcholine vesicles and, by comparison, 86% (37/43) eggs were fertilized by untreated spermatozoa. It seems clear from Table I that cholesterol is responsible for the inhibitory action of the synthetic vesicles, as cholesterol suspensions lacking phospholipid significantly lowered fertilization. Sperm cells suspended in medium containing 0.4 to 4.0 mg cholesterol/

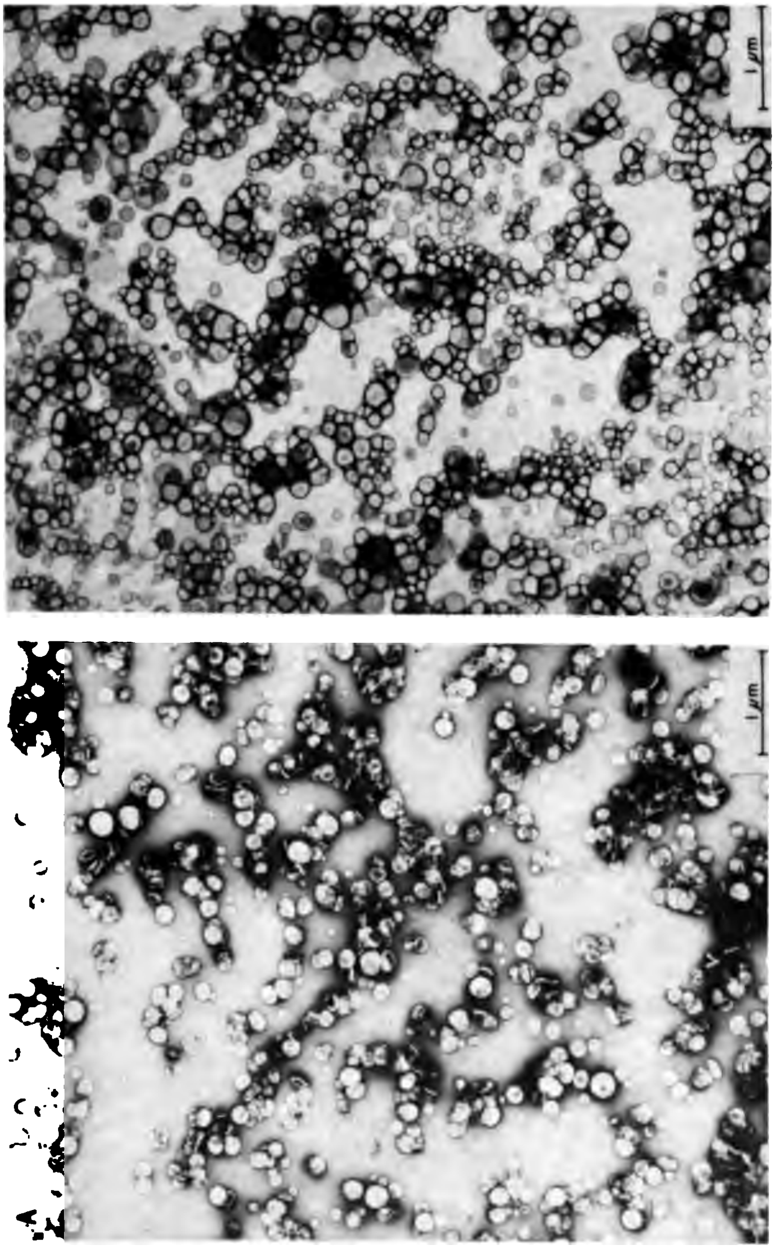

FIG. 1. Electron micrographs of negatively stained vesicles prepared from (A) DPPC and (B) DPPC with 10 (w/w)% cholesterol.

TABLE I. INFLUENCE OF VARIOUS LIPID SUSPENSIONS ON THE FERTILIZING CAPACITY OF RABBIT SPERMATOZOA

Lipid suspension				Eggs			
				Control oviduct		Experimental oviduct	
Composition (w/w)%	Amount (mg/ml)	Number of inseminations	Number of sperm cells (× 10^4)	Total	No. fertilized (%)	Total	No. fertilized (%)
			Oviductal insemination 2 to 4 hr postovulation				
DPPC (100)	10	4	2.5	19	17 (89)	17	12 (71) NS[a]
DMPC (100)	1	4	50.0	24	20 (83)	18	16 (89) NS
				43	**37** (86)	**35**	**28** (18) NS
DPPC (60–80) + cholesterol (10–40) + phosphatidylserine (0–10)	1–2	7, 8[b]	43.0	26	15 (58)	30	3 (10)*
DMPC (60) + cholesterol (40)[c]	1	4	22.5	15	10 (67)	17	4 (24)*
	9–10	9	11.2	46	42 (91)	32	1 (3)*
				87	**67** (77)	**79**	**8** (10)*
Glycerophosphocholine (control)	0.5–1	9	52.0–1.5	28	15 (54)	36	21 (58) NS
Glycerophosphocholine (83) + cholesterol (17) (experimental)							
Cholesterol (100)	0.4	3	3.8	17	17 (100)	10	2 (20)*
	4	3	5.0	13	13 (100)	15	0 (0)*
				30	**30** (100)	**25**	**2** (8)*
			Uterine insemination 5 to 6 hr preovulation				
DPPC (60) + cholesterol (40)	1	6	67.0	21	9 (43)	20	9 (45) NS
DMPC (60) + cholesterol (40)	1	6	20.0–0.5	21	14 (67)	14	9 (64) NS
				42	**23** (55)	**34**	**18** (53) NS

[a] * Statistically significant difference ($P < 0.05$). NS, nonsignificant difference.

[b] Seven control and eight experimental inseminations.

[c] Four inseminations were made in each group with untreated sperm cells in the contralateral oviduct.

ml fertilized 8% (2/25) eggs, while all eggs (30/30) recovered after insemination of untreated spermatozoa were fertilized.

In the presence of 0.5 to 1 mg of lipid/ml of glycerophosphorylcholine and 17 (w/w)% cholesterol (0.085 to 0.17 mg cholesterol/ml) the fertilization rate was 58% (21/36), and this compares with 54% (15/28) fertilization with untreated spermatozoa (Table I). The ineffectiveness of glycerophosphorylcholine and cholesterol in this experiment may indicate the fatty acid side chain of the phospholipid aids in the inhibitory action, presumably by helping to disperse cholesterol, since phospholipid vesicles bearing 0.2 mg of cholesterol blocked fertilization.

Discussion. Suspensions of DMPC and DPPC vesicles containing cholesterol were found to reversibly inhibit fertilization by uterine-capacitated rabbit spermatozoa. The ability of these synthetic vesicles to mimic the action of membrane vesicles from seminal plasma (3) suggests that lipids in the latter are implicated in sperm decapacitation. Cholesterol was shown to be necessary for decapacitation activity and the sterol is known to be present in both classes of vesicles in seminal plasma from rabbits (9).

Concentrations of 1 to 10 mg of lipid/ml were present in the vesicle suspensions used in this work. By comparison, 0.02 to 1.0 mg of protein/ml (approximately 0.014 to 0.43 mg lipid/ml) vesicles from seminal plasma induced decapacitation of rabbit spermatozoa (1, 3). When sperm numbers are taken into account, DPPC–cholesterol vesicles were found to be inhibitory at a concentration equivalent to 5.8 μg of lipid/10^5 sperm cells, and this compares with an effective level of 10 μg of protein/10^5 sperm cells (about 7 μg lipid/10^5 sperm cells) obtained ɔw density seminal plasma vesicles (1). /ed from this perspective, synthetic and ral vesicles have similar potency.

ıosphatidylcholine (DPPC) vesicles been reported to fuse with the plasma ıbrane of living cells at 37° (9). Addi- of cholesterol to the sperm plasma ıbrane of living cells at 37° (10). Addi- aining cholesterol could prevent the ac- me reaction, for the sterol is known to ›it membrane fusion (11, 12). The ac- ' of some membrane-associated en- :s could also be influenced by the pres- of cholesterol. Decreased fluidity re- ıg from a partial restriction of motional lom among fatty acid side chains in liq- crystalline phospholipid bilayers has suggested as the basis of the action by :sterol (12, 13).

ıese experiments obviously do not ex- : the possibility that phospholipid sus- ions containing cholesterol, that have ɔeen dispersed as vesicles, could also ›it sperm-fertilizing capacity. In this ection, it may be noted that presatura- of albumin with cholesterol prevented ization of rat eggs *in vitro* (14). The :nt findings give added credence to the :stion that addition of lipid (choles-) to the sperm plasma membrane could ıplicated in sperm decapacitation (15). ›PPC, unlike DMPC, has a critical tem- ure for "gel to liquid" transition in s of the phospholipid (13) that is above ological temperature, it may be antici- l to exert a stabilizing influence on branes. The inability of DPPC vesicles capacitate rabbit spermatozoa possibly ts from rapid hydrolysis by sperm phos- pase (16) following incorporation.

he reversible inhibition of sperm-fertil- capacity observed in these experiments uivalent to decapacitation by seminal ıa, then involvement of polypeptide glycoprotein in sperm decapacitation 18) perhaps indicates alternative (non- ways of inducing decapacitation exist. ıer experimentation should help clarify gnificance of this possibility.

nmary. Suspensions of dimyristoyl and nitoyl phosphatidylcholine vesicles ıg 10 to 40 (w/w)% cholesterol in- d the fertilizing ability of uterine-capacitated rabbit spermatozoa at concentrations of 1 to 10 mg of lipid/ml. Recovery of fertilizing ability by treated sperm cells was observed following insemination into the uterus 5 to 6 hr before ovulation. Vesicles lacking the sterol were not inhibitory under the conditions employed. Suspensions of cholesterol (0.4 to 4 mg of sterol/ml) without phospholipid, in contrast, inhibited fertilization. Implication of cholesterol in sperm decapacitation by seminal plasma membrane vesicles is discussed in terms of these results.

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Fate of Intraintestinal Thymocytes Labeled with ^{125}Iododeoxyuridine or Tritiated Thymidine[1, 2] (39375)

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Migration of blood lymphocytes into the lumen of the small intestine has been reported (1, 2). The origin of these lymphocytes remains unknown; however, the thymus has been proposed as a possible source (3). Following local thymic labeling with a radioactive DNA precursor, a slight accumulation of radioactivity has been detected in the intestine and its contents (4). If migrants are not catabolized in the intestine, the enteric loss of these labeled thymocytes might be quantitated by measuring fecal radioactivity. To our knowledge, quantitative data on the kinetics of breakdown in the intestinal lumen of cells labeled with radioactive DNA precursors are not available. The present experiments were designed to evaluate the fate of donor thymocytes labeled with either ^{125}iododeoxyuridine (^{125}IUdR) or tritiated thymidine (^{3}HTdR) and injected into the small intestine of recipient mice.

Materials and methods. Random bred, female Swiss albino mice of the Hale-Stoner strain, 8 to 12 weeks old, were used as donors and recipients. Two days before intrajejunal (ij) injection of cells labeled with ^{125}IUdR, KI (0.5 mg/ml) was added to the drinking water of recipients (4). The intestinal flora of one group of conventional mice was removed by adding high doses of nonabsorbable antibiotics to the drinking water and housing under sterile conditions for 1 week as reported by van Bekkum and coworkers (5).

5-^{125}Iodo-2′-deoxyuridine (New England Nuclear, Boston, Mass.; sp act > 200 Ci/mmole; 203 to 480 μCi/ml) was diluted with normal sterile saline (9 mg/ml) to contain 100 μCi/ml. Donor mice were labeled by two to four iv or ip injections at 6- to 12-hr intervals (total dose per mouse 20, 40, or 70 μCi). Thymidine (methyl-^{3}H; Schwarz/Mann, Orangeburg, N.Y.; sp act 1.9 Ci/mmole; 1 mCi/ml; diluted with normal saline to contain 200 μCi/ml) was injected ip into donor mice at 8-hr intervals (eight injections, total dose per mouse 160 μCi). All donors were killed by ether overdose 24 hr after the last injection. Donor thymi were minced in Hanks' balanced salt solution (HBSS, without Ca and Mg; Gibco, Grand Island, N.Y.) to which 4 mg/ml of Na_2 EDTA had been added. The cell suspensions were forced through 22-gauge needles, filtered with nylon mesh, washed twice in 50 ml of HBSS and concentrated to contain about 1 to 9 × 10^7 cells per dose. This corresponded to approximately 3000 and 6000 cpm for ^{125}I and ^{3}H, respectively. Trypan blue-positive cells were enumerated (6). In a control experiment, the suspended cells were killed by repeated freezing and thawing. Mice were anesthetized by ip sodium pentobarbital (4). The proximal end of the recipient jejunum was exposed through a small (5 mm) incision, and 0.05 ml of the cell suspension was injected ij using a 30-gauge atraumatic needle. The wound was closed and the animals placed in individual plastic cages (4). Recipients were killed at the time indicated in the figures. The intestine (pylorus to anus) and its contents, carcass, feces, urine, and in some instances spleen and thymus were collected and kept at 4° prior to counting ^{125}I-activity in a well-type scintillation counter (Nuclear Chicago, 1185 series). Aliquots of thymocytes labeled with ^{3}HTdR and tissues of recipient animals were subjected to formalin

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[2] The research described in this report involved animals maintained in animal care facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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extraction of acid-soluble radioactivity (ASR) (4). Total tritium activity of tissues and formalin extracts was determined by scintillation counting following oxidation (TriCarb oxidizer, Packard).

Results. Thymocyte suspensions were essentially monodispersed and contained less than 10% trypan blue-positive cells. More than 90% of the radioactivity of the suspension was cell-bound and not extractable with formalin. In the control experiment, more than 95% of the morphologically recognizable cells were stained by trypan blue.

Following injection of thymocytes labeled with ^{125}IUdR, the decline of radioactivity of the recipient intestine and its contents could be described by two exponential components, with a high rate of activity loss during the first 5 hr (Fig. 1). Viability of the cells prior to injection did not significantly affect the rate of ^{125}I-activity loss, i.e., 35.6, 9.9, and 4.5% of the intestinal activity present at 0 hr remained 1, 3, and 5 hr following injection of thymocytes killed by freezing and thawing. The bulk of the injected ^{125}I-activity lost from the intestine was recovered in the urine, the ^{125}I-activity curve of the latter essentially mirroring the intestinal activity. Less than 3% of the initial activity appeared in the feces.

During the first 4 to 5 hr following ij injection, intestinal loss of ^{125}I-activity in recipients pretreated with antibiotics (Fig. 2) was nearly as rapid as that observed in conventional mice. Thereafter, a slower rate of loss was observed; however, values at 24 hr postinjection were not significantly different from those seen in conventional mice. The proportion of cumulative ^{125}I-activity in the feces was somewhat higher than in conventional animals. The sum of activities recovered in urine, feces, intestine, and the remainder of the body during the first 24 hr postinjection accounted for the near-totality of the initial radioactivity in both groups.

Tritium activity was lost from the intestine initially as rapidly as ^{125}I-activity; 3 hr after ij injection approximately 10% of the total initial ^{3}H-activity remained in the intestine (Fig. 3). Thereafter, the loss of ^{3}H-activity was slow. The relative activity of the intestine including ASR 24 hr postinjection was about 15 times higher than the corresponding values for ^{125}I.

Discussion. Thymocytes labeled with ^{3}HTdR or ^{125}IUdR appeared to be rapidly catabolized in the small intestine. Elimination of ^{125}I-activity in the feces was, however, minimal. It was observed earlier that the fecal radioactivity following systemic labeling with ^{125}IUdR remained low even though the major part of activity was lost from the intestine presumably through sloughing of labeled epithelial cells into the lumen. The bulk of the label was apparently

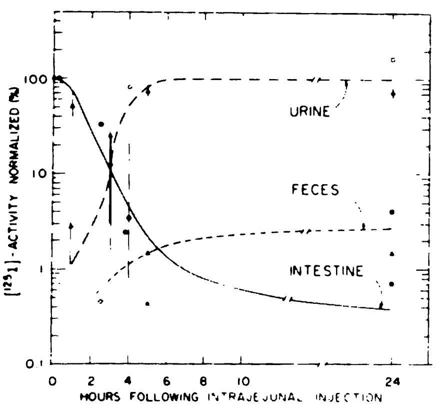

FIG. 1. Normalized ^{125}I-activity (percentage of intestinal activity at 0 hr, ±SD) versus time (0, 1, 2.5, 3, 4, 5, 10, and 24 hr) following ij injection of thymocytes labeled with ^{125}IUdR. Triangles (average of three mice) and circles (average of two mice) represent data from two separate experiments. The curves were fitted by eye. ●▲ = Intestine and contents; ○△ = cumulative urinary activity; ◐△ = cumulative fecal activity.

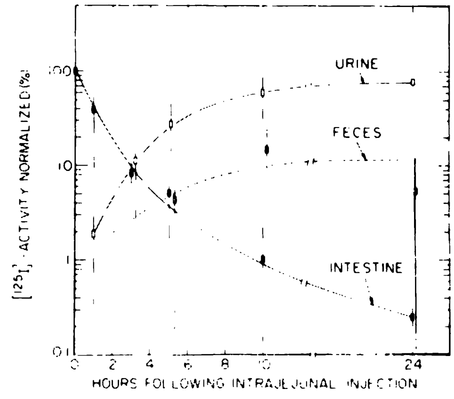

FIG. 2. Recipients were pretreated with antibiotics. ^{125}I-activity (percentage of intestinal activity at 0 hr, average of four to five mice, ±SD) versus time following ij injection of thymocytes labeled with ^{125}IUdR. Symbols analogous to those used in Fig. 1.

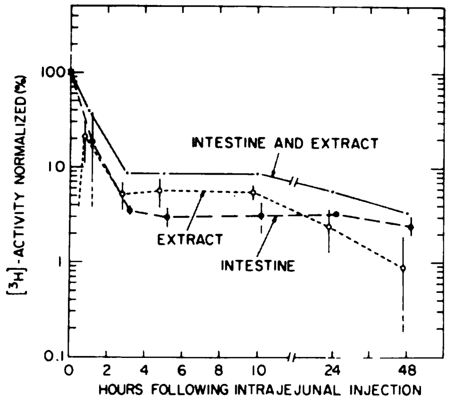

FIG. 3. Normalized ^{3}H-activity (percentage of intestinal activity at 0 hr, average of five mice, ±SD) of intestine and contents = ●, formalin extracts = ○, and sum of activities of intestine and formalin extracts = •, versus time (0, 1, 3, 5, 10, 24, and 48 hr) following ij injection of thymocytes labeled with ^{3}HTdR.

reabsorbed and could be recovered in the urine (4).

It is unlikely that damage due to radiotoxicity to thymocytes prior to ij injection would significantly alter the interpretation of the results, since the proportion of ASR as well as the number of trypan blue-positive cells in the suspensions remained low. Presumably, the thymocytes were killed very rapidly, since the breakdown of dead cells occurred essentially at the same rate as that of viable counterparts.

Catabolism of viable or of dead cells and excretion of breakdown products was initially very rapid. Similarly, Kotani and coworkers (1) found a rapid increase in ^{3}H-activity of portal blood and thoracic duct lymph following injection of lymphocytes labeled with ^{3}HTdR into the small intestine. In portal vein serum, ^{3}H-activity was associated with thymine, thymidine, and thymidylic acid (1).

Selective removal of ^{125}I from intact DNA labeled with ^{125}IUdR has not been demonstrated *in vivo* (7). The marked similarity of the initial phase of activity loss following ij injection of cells labeled with ^{125}IUdR or ^{3}HTdR is compatible with this observation, since cell death followed by degradation of DNA labeled with ^{3}HTdR is considered the only way in which ^{3}H could be lost from a tagged cell (8). Ingested nucleic acids are degraded in the small intestine by intestinal and pancreatic nucleolytic enzymes to yield absorbable nucleosides, free bases and inorganic phosphate (9-11), and DNA, normally present in the food, has not been listed among the normal constituents of human feces (10).

Catabolites of DNA from ^{3}HTdR-labeled lymphocytes injected either intraintestinally (1) or intravenously (12) are locally and systemically reutilized by cell renewal systems in the body. In the present experiment, thymus and spleen of recipient mice showed very low, but definite ^{3}H-activity 24 hr following ij injection of labeled thymocytes, whereas no ^{125}I-activity could be detected in these organs. Local intestinal utilization, and hence reutilization, may be less than systemic utilization: the intestinal uptake of ^{3}HTdR and ^{125}IUdR, following intraintestinal injection is about 50 and 85%, respectively, less than following ip administration of the same isotope dose (13). This difference may be due to a poorer intestinal uptake of ^{125}IUdR and/or more active catabolization in the intestine or liver (7, 13).

Whereas substantial ^{3}H-activity not associated with DNA persists for long periods of time following labeling with ^{3}HTdR (14, 15), ^{131}I not incorporated into DNA is rapidly lost following iv injection of ^{131}IUdR (7). Therefore, the slower phase of activity loss following ij injection of ^{3}HTdR-labeled as compared to ^{125}IUdR-labeled thymocytes may indicate a slower loss of ^{3}H-activity not associated with DNA and/or a greater reutilization.

The intestinal flora did not appear to play a significant role in the rapid catabolism of labeled cells, because no marked difference was observed between conventional animals and mice pretreated with antibiotics that effectively sterilized the gut. Similarly, the rapid intestinal hydrolysis of nucleotides *in vitro* was not altered by addition of antibiotics to the system (16).

It is concluded that cells labeled with radioactive DNA precursors may be detected in the intestinal lumen only for a short time; additional techniques such as intestinal perfusion may be required to disclose the presence of labeled migrants in the gut.

Summary. A suspension of thymocytes labeled with ^{125}IUdR or ^{3}HTdR was injected into the jejunum of mice. The bulk of the adioactivity disappeared within few hours rom the intestine and was recovered princi- ally in the urine. This indicated a very apid breakdown of labeled thymic cells, eabsorption and subsequent elimination of he tracer in the kidney. In mice injected ith cells labeled with ^{3}HTdR, the initial apid loss of radioactivity was of shorter uration, and slower during the second hase, presumably due to more extensive eutilization and/or prolonged persistence f acid-soluble radioactivity. Pretreatment f the recipients with antibiotics did not sig- ificantly reduce the rate of radioactivity oss.

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Effects of Calcium and Phosphatidyl Serine in Rat Mast Cell Reaction to Dextran (39376)

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Dextran releases histamine from normal rat mast cells in a reaction that resembles anaphylaxis (1-4). The reaction requires Ca^{2+} in the medium. Using Sprague-Dawley rat mast cells we found that exogenous Ca^{2+} was necessary to initiate the reaction (5), in contrast to its requirement only in the final stage of anaphylactic histamine release from human leukocytes (6). Substantial release from mast cells of most rat strains by dextran or antigen also requires the addition of phosphatidyl serine (PS), as shown by Goth *et al.* (2) and confirmed by others (3, 7, 8). Release from Wistar rat cells does not require added PS (9), however, nor does the human leukocyte reaction (6). The mechanism of histamine release evidently varies in some degree, depending on the type of cells and the releasing agent employed. The present study was made to explore further the roles of Ca^{2+} and PS in histamine release from Sprague-Dawley rat mast cells by dextran.

Methods and materials. Mixed peritoneal cells were obtained from 300-g male Sprague-Dawley rats as previously described (3, 10). The peritoneal cavity was irrigated with 15 ml of normal buffer, or (sometimes, if some of the cells were to be used in low-Ca solutions) with Ca-free buffer, with 5 μg/ml of heparin. The normal buffer was Krebs-Ringer phosphate, pH 7.0, with 1.0 m*M* Ca^{2+}, no Mg^{2+}, and 1 mg/ml of crystalline bovine albumin. For each experiment, the irrigating solution from two or three rats was collected in an ice-cold tube. The cells were sedimented in one or more portions, and resuspended in buffer solution(s) (15 ml/donor rat), without heparin. Replicate 1.5-ml samples[1] of the pooled cells were pipetted into siliconized tubes, into final volumes of 2.5 ml of the desired solutions, and equilibrated for 10 min at 25°. Histamine release from the cells was induced by adding dextran, together with a small amount of PS, in 1.0 ml of the appropriate buffer solution, and incubating for 15 min at 25° (10). Final concentration of dextran (Pharmacia Fine Chemicals, average mol wt 2×10^{6}) was 1.0 mg/ml, and of PS (Nutritional Biochemicals Corp) 7 μg/ml, except when stated otherwise. PS was added to the control tubes without dextran, and was used in all experiments unless there is a statement to the contrary. In a few experiments, the cells were obtained from rats that had been immunized with egg albumin (EA) and pertussis vaccine (12), and EA 10^{-4} mg/ml (with PS) instead of dextran was used as the releasing agent. Except for the differences in treatment being studied, all cell samples in each experiment were treated alike. Release from duplicate samples by dextran differed, on average, by only 2 ± 2% of the respective release values (n = 27). Histamine determinations were made fluorometrically (16) as previously described (3). Degree of histamine release was assessed by dividing the amount released by the amount released plus the amount retained in the cells.

Results. Effects of Ca^{2+} on histamine

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[1] Counts on samples of mixed peritoneal cell suspensions (11) showed about 10^{5} mast cells per 1.5 ml, and histamine content was about 2 μg/1.5 ml. The number of cells used per tube was not critical, however, as diluting the suspension 10-fold did not affect the degree of histamine release by dextran (3). Neither did adding 3×10^{-6} *M* histamine with the dextran (3) or adding dextran in the form of the supernatant solution from a previous release reaction (12) affect release. Ficoll- or albumin-gradient fractionation (13) often left the mast cells poorly responsive (3), not corrected by adding back the other cells. Therefore, after it had been established that virtually all the histamine was in the mast cells (3), and that pure mast cells responded in the same manner as unfractionated cells (7, 13-15), we used the mixed cells without fractionation.

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Dextran released 0 ± 2% of the
: from cells that had been trans-
ı Ca-free medium, compared with
% in medium containing 1.0 m*M*
:an ± SD, *n* = 4). For comparison,
se values obtained with cells from
ed rats by using the specific antigen
e releasing agent, were 13 ± 11%
ree buffer and 31 ± 19% with 1.0
(*n* = 4). Thus, only the release by
vas completely dependent on exog-
ı$^{2+}$.[2] The levels of Ca^{2+} (1.0 m*M*)
) μg/ml) used in these experiments
determined to give approximately
release under the experimental
s. Changing the pH inversely af-
: required level of Ca^{2+}. Ca^{2+} levels
M or less were completely ineffec-
supporting release by dextran.
as not used in conjunction with the
nedium.

ınd cell desensitization. The de-
period-2 histamine release by dex-
h 1 m*M* Ca^{2+}) due to the cells
:en exposed to dextran (instead of
ı the presence of various levels of
eriod 1, is shown in Table I (last
The decrease, reflecting the de-
:ll desensitization to dextran, was
:n period-1 Ca^{2+} level was 0.1 m*M*
With 0.3 m*M* Ca^{2+} in period 1,
ation was considerable but incom-
l with 1.0 m*M* Ca^{2+} it was virtually
as previously shown (5).

:e of Ca^{2+} on cell integrity. Simply
ıe cells for 15–30 min in Ca-free
ıt 25° caused them to leak abnor-
ınts of histamine (14 ± 5%, com-
h 2 ± 1% in normal medium, *n* =
they could not be returned com-
their original state of responsive-
dding back Ca^{2+}. Table I shows
ibration of the cells with low-Ca
period 1 caused them to release
nine after Ca^{2+} had been restored
(period 2, first column). In three additional experiments, the mean reduction in release due to prior exposure of the cells to Ca-free medium at 25° was 35% of respective release values. Effects of exposure to low-Ca solutions at 37° were greater than at 25°; effects at 0° were relatively slight.

TABLE I. EFFECT OF Ca^{2+} CONCENTRATION ON HISTAMINE RELEASE AND CELL DESENSITIZATION BY DEXTRAN (1 mg/ml).[a]

Period 1		Period 2: Ca^{2+} 1.0 m*M*	
		Histamine release by dextran (net)	
Ca^{2+} Concn (m*M*)	Histamine release by dextran (net) Percentage of cell histamine	After buffer in period 1 Percentage of cell histamine	Decrease due to dextran in period 1[b] Percentage of cell histamine
0	1	11	0
0.01	0 (1)[c]	13 (15)	2 (1)
0.03	0 (0)	13 (18)	0 (3)
0.10	0 (1)	18 (24)	1 (3)
0.30	21 (32)	24 (32)	18 (26)
1.00	60 (39)	28	28

[a] Replicate samples of pooled cells were equilibrated with buffers with various Ca^{2+} levels. Then representative samples were treated with dextran (for 10 min at 25°) in period 1. Net histamine release by dextran (over that by the corresponding buffer alone) was assessed from determinations on the supernatant solutions. The same cell samples (whether exposed only to buffers or to dextran solutions in period 1) were washed twice with cold Ca-free buffer, equilibrated with 25° 1.0 m*M* Ca^{2+} buffer, and challenged (for 15 min) with dextran in period 2. PS used in all tubes.

[b] Compared with values in column immediately to the left.

[c] Results of a second experiment are shown in parentheses.

Time required to remove effect of Ca^{2+}. Duplicate cell samples that had been harvested in low-Ca buffer and equilibrated with buffers of various Ca^{2+} levels were treated, respectively, with dextran dissolved in the same buffer used for the cells and with dextran in Ca-free buffer. The additions of dextran in Ca-free buffer diluted the Ca^{2+} in the cell medium by 40%, and in each case released the amount of histamine expected with the final (diluted) Ca^{2+} level, rather than that expected with the original level, as shown in Fig. 1. Similarly, with cells harvested in 1.0 m*M* Ca^{2+}, and using a volume of dextran that gave 60% dilution (not shown), the Ca^{2+}-dilution was in large de-

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/e recently compared the Ca^{2+} and PS de-
f histamine release from Sprague-Dawley
ls by the following agents: dextran, antigen,
ıncanavalin A, the ionophore A23187, so-
compound 48/80, protamine, and polyly-
ıdence of the different agents on both Ca^{2+}
e medium differed greatly, but only dextran
tely dependent on exogenous Ca^{2+}.

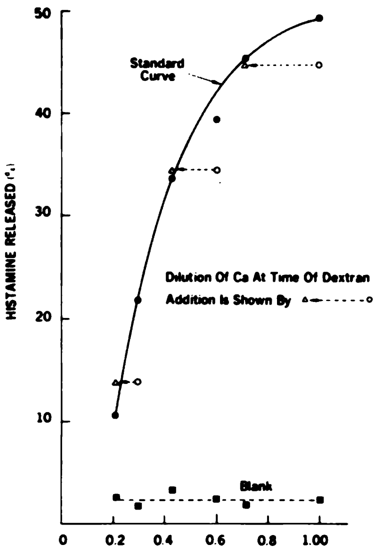

FIG. 1. Effect of diluting Ca^{2+} in cell medium at time of adding dextran. Ca-free dextran solution was added to cell suspensions containing three different levels of Ca^{2+}, and the resulting histamine release in each case is plotted vs the original Ca^{2+} level of the medium (○), and vs the Ca^{2+} level after the Ca-free dextran addition (△). In each case, the plot using the final Ca^{2+} levels falls very close to the standard curve (determined by adding dextran solution containing the same Ca^{2+} level as the cell suspension in each case). The blank values (■) show release by buffer alone.

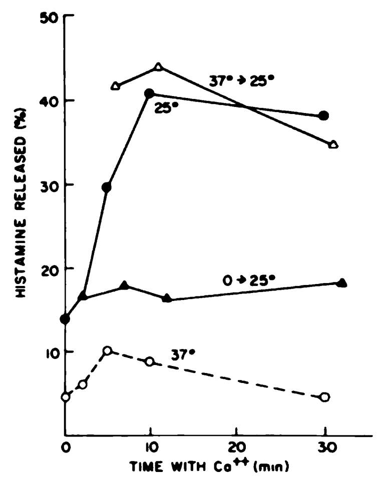

FIG. 2. Effect of preincubation time of cells with 1.0 mM Ca^{2+} on histamine release by dextran. The cells had been in Ca-free buffer at 25° for 15–30 min. Results are shown for preincubation of the cells with Ca^{2+} and challenge with dextran at 25° (-●-); preincubation and challenge at 37° (-○-); preincubation at 0° and challenge after 2 min at 25° (-▲-); and preincubation at 37° and challenge after 2 min at 25° (-△-). Mean results of three 25°-experiments and of four 37°-experiments are shown. Individual results in each group were similar in trend.

gree (usually about 75%) reflected in the resulting decrease in histamine release. Even when cells were concentrated in a small volume of 1.0 m*M* Ca^{2+} buffer and treated with Ca-free dextran in relatively large volume, so that the Ca^{2+} level of the medium was thereby reduced below 0.1 m*M* (a level at which no release occurs from equilibrated cells), virtually no net release occurred (2 expts.). Since histamine release by dextran at 25° starts within 30 sec and is largely completed in 2 min (12), these results show that dilution of Ca^{2+} in the medium promptly altered cell responsiveness almost to the full extent predicted by the degree of dilution (in contrast to the slowness of restoration of the Ca^{2+} effect considered below).

Time required to restore Ca^{2+} effect. Cells that had been in Ca-free buffer for 15–30 min at 25° released relatively small amounts of histamine when Ca^{2+}, 1 m*M*, and dextran were added to them simultaneously (Fig. 2, zero time). A period of preincubation of the cells with the Ca^{2+} was required, before addition of dextran, in order for dextran to produce maximal release. At 25°, a 10-min preincubation was required (Fig. 2). If the cells were preincubated with Ca^{2+} at 0°, and then changed back to 25° for 2 min before challenge with dextran, there was virtually no increase in histamine release as preincubation time increased. When preincubation was at 37°, maximal release at 37° was reached after only about 5 min, although release was small, as always at 37° (10). If the 37°-preincubated cells were changed back to 25° for 2 min before dextran was added, release was greater, and the earlier peak observed at 37° was in part maintained. Thus, a significant part of the effect of Ca^{2+}, depended on the Ca^{2+} reaching, by a temperature-dependent process, cell sites that were not immediately available.

Effects of other cations. In Ca-free medium, Sr^{2+}, Ba^{2+}, and Mg^{2+} in concentrations up to 10 m*M* each consistently failed to replace Ca^{2+} in supporting histamine release by dextran (Table II). Spontaneous

I. EFFECT OF VARIOUS DIVALENT CATIONS
STAMINE RELEASE FROM THE CELLS BY
DEXTRAN.[a]

lded	Concn (mM)	Histamine release by dextran (net)[b] (% of cell histamine)
:		1 ± 0
	1.0	1 ± 0
	3.0	0 ± 0
	10.0	1 ± 0
	1.0	1 ± 1
	3.0	1 ± 0
	10.0	0 ± 0
	1.0	0 ± 1
	3.0	1 ± 1
	10.0	−1 ± 2
	1.0	23 ± 15
	3.0	14 ± 10
	10.0	6 ± 3

lutions were Ca-free except where Ca^{2+} is oncn 10 μg/ml. Tris-HCl, 18 mM, was sub- phosphate of normal buffer. Cations were hlorides. Cells were preincubated with salt r 10 min before dextran was added.

of release by dextran over that by the corre- lt solution alone, ± SD. Mean results of nents, each made with replicate samples of s, are shown.

f histamine into the medium (with- an) was unaffected or decreased by n additions. When used in conjunc- various Ca^{2+} levels, Mg^{2+} 1.0 mM out effect or somewhat inhibitory e by dextran (not shown). Mn^{2+}, mM, did not support release by vithout Ca^{2+}, and strongly inhibited se by dextran with Ca^{2+}. Na^{+} and not required for release, as satisfac- ase was obtained in 0.18 M Tris- fer with no other cations present a^{2+}. Ouabain, 10^{-5}–10^{-3} M, was ffect on release by dextran in nor- r and in K-free buffer.

ge of release by EDTA or glucose, tion of cell medium. As shown in dding EDTA (3 mM) can stop his- elease (in normal buffer) short of n, if the EDTA is added to the in 1 min after the addition of dex- ly addition of glucose (4 mg/ml) inished the amount of histamine as did 15-fold dilution of the cell- nixture with normal buffer. The lts were obtained when the added contained 10 μg/ml of PS, show- ilution of PS was not the basis of age.

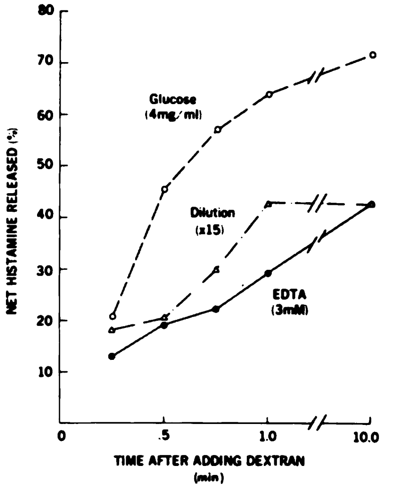

FIG. 3. Reduction in histamine release by dextran due to addition of EDTA or glucose, or to 15-fold dilution of cell suspension with normal buffer. Different cell preparations were used in the three experiments. Additions were made at the indicated times after addition of dextran, and histamine release was determined after 15-min incubation with dextran.

Effect of PS on histamine release. PS was used in the experiments discussed thus far in concn of 7–10 μg/ml, which was nearly as effective as much higher levels. PS alone did not release histamine. It increased release by dextran from 7 ± 5% up to 52 ± 10% (n = 8), as expected from previous results. We found the effect of PS to be virtually the same, whether it was added to the cells 10 min before dextran or together with the dextran (49% vs 50%, n = 3). Likewise, adding the PS 30–45 min before dextran did not further increase release. Thus, the PS effect was very rapidly established, reasoned as follows. Without PS, dextran releases little histamine, but still rapidly desensitizes the cells (by about 50% in 2 min) (12, 17). Therefore, dextran added with PS would have released much less histamine than dextran added 10 min or longer after PS, if PS had not become effective very rapidly.

Washing the cells twice with buffer after adding PS but before adding dextran, decreased release by 50–90% (compared with release from similarly washed cells to which PS was added after washing, rather than before). This indicated that some of the cell-associated PS could be washed away. We attempted to substitute phosphatidyl glycerol (which does not seem to have been tested) for PS in the release reaction, but found it without definite effect in concns up to 28 μg/ml.

Interaction of Ca^{2+} and PS. As Ca^{2+} concn was varied from 0.2 m*M* up to 3 m*M* in different tubes, histamine release by dextran increased to a maximum and then declined, whether or not PS was used. Use of PS (7-14 μg/ml) in the experiments increased release, as already noted, but also shifted the peak of release to higher Ca^{2+} levels. Thus, whereas release without PS was greater at 0.5 m*M* than at 1.0 m*M* Ca^{2+} in five of six experiments (mean of 11% vs 9%, $p = 0.15$), release with PS was greater at 1.0 m*M* Ca^{2+} in all cases (mean of 56% at 0.5 m*M* vs 60% at 1.0 m*M*, $p = 0.03$), suggesting that the addition of PS increased the level of Ca^{2+} required to give maximum release. Binding of Ca^{2+} by the PS (mole for mole) would not have significantly changed the Ca^{2+} level in the medium.

Discussion. This study showed that histamine release from Sprague-Dawley rat mast cells by dextran was completely dependent on exogenous Ca^{2+} in the medium, in contrast to anaphylactic release from the same cells. Others have also noted the incomplete dependence of anaphylactic release on Ca^{2+} in the medium (18). We demonstrated that the degree of cell desensitization by dextran increased progressively as Ca^{2+} in the medium increased, in amplification of our previous study (5). In the virtual absence of Ca^{2+} in the medium, spontaneous leakage of histamine from the cells increased, and the release response could not be entirely restored to normal by adding back Ca^{2+}.

We found no effective substitute for Ca^{2+} in the release reaction, in contrast to the finding of Foreman and Mongar (19, 20) that Sr^{2+} (1-10 m*M*) and Ba^{2+} (5-10 m*M*) considerably increased release from their cells by antigen without Ca^{2+}. Also, we found that Sr^{2+} and Ba^{2+} (and Mg^{2+}) usually decreased (rather than increased) spontaneous histamine leakage. Whether these differences in results are due to differences in rat strain or releasing agent, or to subtle differences in techniques, is not clear.

PS enhances release from rat mast cells by increasing the rate of release, rather than by prolonging the duration of release (7, 8) by decreasing the rate of desensitization (12, 17). Although histamine release and cell desensitization by dextran are completely dependent on Ca^{2+} in the medium, release in small degree and desensitization at the usual rate occur without PS (12, 17). Several workers (2, 8, 15) have postulated that PS enhances release by activating Na^+, K^+-ATPase, although they found, as we did in this study, that ouabain did not affect the release (8, 17).

We previously suggested that (in addition to other actions of Ca^{2+}) Ca^{2+} and PS might act together at the cell surface, as the dextran receptor function in Sprague-Dawley cells seemed to depend on exogenous Ca^{2+} (5). Others have considered a similar mechanism in anaphylactic release from rat mast cells (19). Possibly the present observations support this concept. Although Ca^{2+} required 10 min to become maximally effective in supporting release when added back to Ca-deficient cells (and its immediate effect was small), dilution of Ca^{2+} in the cell medium was promptly and substantially reflected in a decrease in release by dextran. Likewise, addition of PS to the cell medium was immediately fully effective in increasing release. Thus, both Ca^{2+} and PS produced obligatory effects at quickly accessible cell sites. Interaction of the two factors was shown by their synergism, and probably by an effect of PS on the Ca^{2+} level required for maximum release by dextran. With cells that do not require PS for release, the effects of Ca^{2+} may differ from those observed here (21).

The delayed and incomplete restoration of histamine release associated with the readdition of Ca^{2+} to Ca-deficient Sprague-Dawley cells, complicates experiments involving exposure of cells to Ca-free solutions, such as the present experiments, and our previous studies of cell activation and desensitization without Ca^{2+} (5). However, other investigators, using cells from other sources, do not seem to have encountered these problems (6, 17, 18).

The presently observed stoppage of dextran-induced histamine release by EDTA is not surprising (except perhaps for the promptness of the effect), assuming that Ca^{2+} is required in the final stage of the release, as it is in the human leukocyte-antigen reaction (6, 22). The stoppage by adding glucose (presumably due to competitive inhibition of dextran binding (1)) and by diluting the cells (probably due to the reduction in dextran concn) was unexpected, however, by analogy with the human leukocyte reaction, as that reaction proceeds in the absence of the releasing agent once the cells have been activated (6).

Summary. Histamine release from Sprague-Dawley rat mast cells by dextran was completely inhibited by the absence of exogenous Ca^{2+} (in contrast to release from the same cells by antigen). Also, spontaneous leakage of histamine from the cells increased in the absence of Ca^{2+}, and cell responsiveness was not completely restored by readding Ca^{2+}. We found no effective substitute for Ca^{2+} in the release reaction. Ca^{2+} was not maximally effective immediately when added back to Ca-deficient cells, but almost the full effect of diluting Ca^{2+} in the medium (which decreased release) and of adding PS (which increased release) were very rapidly established, suggesting that both Ca^{2+} and PS might act (in part) at superficial cell sites. Release from activated cells could be stopped short by adding glucose or by diluting the cell-dextran mixture with normal buffer, as well as by adding EDTA, which deserves further study.

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Radioimmunoassay (RIA) of Relaxin in Sera of Various Species Using an Antiserum to Porcine Relaxin (39377)

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Recent work from our laboratory led to the development of a radioimmunoassay (RIA) for porcine relaxin utilizing [^{125}I]polytyrosyl relaxin (1). Addition of tyrosine to the relaxin molecule proved to be necessary as the purified native hormone does not contain tyrosine or histidine (2) and fails to iodinate when subjected to the Hunter and Greenwood method (1). An impure relaxin extract was used by other workers who claimed successful iodination of the hormone (3).

We have previously demonstrated cross-reactions of antisera to porcine relaxin with relaxin-containing tissues of several species including rats, mice, chickens, whales, and human beings (4). Because of this broad range of immunological cross-reactivity of relaxin from different sources, it was of interest to develop a RIA for determination of blood levels of relaxin in species other than the pig. We have now assayed relaxin levels throughout pregnancy in rats, mice, and guinea pigs, and near term in dogs and primates.

Materials and methods. Radioimmunoassay. The relaxin RIA has been modified since the publication of the original report (1).

Reagents. Two fractions of porcine relaxin prepared according to the procedure of Sherwood and O'Byrne (2) were used in the development of the method. A moderately pure fraction (G-50:1000 U/mg) was used for immunization and a highly purified fraction (CM-A + B: 2500 to 3000 U/mg) was used for standards and preparation of polytyrosyl-relaxin. The assay was equally sensitive and linear when we used a purified relaxin prepared by a different method and kindly supplied by Dr. Christian Schwabe, Medical College of South Carolina. Thus, the antigenic determinants of porcine relaxin do not appear to be altered by differing routes of biochemical purification. However, reduced-alkylated relaxin as well as isolated α and β subunits of relaxin did not compete with [^{125}I]polytyrosyl-relaxin for binding sites on the antibody. Although relaxin precursors and/or metabolic products may be immunoreactive, these observations suggest a requirement for the overall structure of two peptide chains connected by disulfide bonds for antibody recognition. Polytyrosyl-relaxin, [^{125}I]polytyrosyl-relaxin, and relaxin antisera were prepared as previously described (1). Goat anti-rabbit gamma globulin was purchased from Antibodies Incorporated, Davis, California.

RIA procedure. 1. One-hundred microliters of relaxin standard solutions [10 to 2500 pg relaxin in phosphate buffered saline (PBS = 0.01 *M* sodium phosphate, pH 7.0, 0.14 *M* sodium chloride)–1% egg albumin] plus a volume of control serum or plasma (male or castrated) equivalent to that of the unknown samples were added to standard curve tubes.

2. Unknown serum or plasma samples were added to other tubes.

3. Sufficient PBS–1% egg albumin was added to each tube to bring the volume to 500 μl.

4. One-hundred microliters of [^{125}I]polytyrosyl-relaxin in PBS–1% egg albumin (30,000 to 60,000 cpm) were added to each tube.

5. One-hundred microliters of relaxin antiserum diluted in 0.05 *M* EDTA-PBS containing 6% male rabbit serum were added to each tube. (A 1:50,000 working dilution of our antiserum binds 30–40% of the [^{125}I]polytyrosyl-relaxin when no competing unlabeled relaxin is present in the tube).

6. The tubes were then incubated at 4° for 24 hr.

7. Antibody-bound [^{125}I]polytyrosyl-relaxin was precipitated by one of the following two methods. (a) Double-antibody: Two-hundred microliters of diluted goat

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it gamma globulin (50 μl + 150 μl
re added to each tube. The tubes
ıbated at 4° for an additional 24 hr
centrifuged. (b) Polyethylene gly-
sufficient volume of 25% w/w
solution of polyethylene glycol
ıx 6000) at 4° was added to each
ıake the final concentration of the
12.5% (5). The tubes were shaken
centrifuged. Polyethylene glycol
es gamma globulin (5) and thus
economical and fast method for
g antibody-bound hormone from
ıone. However, as the volume of
sayed is increased the number of
ic counts precipitated by polyeth-
col is increased, thereby reducing
tivity of the assay. When relaxin
e so low that more than 100 μl of
plasma were needed for a measur-
onse, the double-antibody proce-
me the method of choice. This was
he case with the samples of serum
from pregnant women.

supernatants were aspirated and
pitates were counted in an auto-
ımma counter (Packard Auto-
pectrometer).

dards and unknowns were assayed
te. Logit and log transformations
ısed to obtain linear dose response

*porcine relaxin added to sera of
cies.* Standard curves were con-
ısing known amounts of porcine
lded to serum or plasma obtained
rats and ovariectomized women.
relaxin standards were added to
mples of pooled plasma obtained
iectomized women then incubated
24 hr prior to RIA to determine if
rum contained a "relaxinase" or
destroyed the hormone.

relaxin was injected im in a ba-
blood samples were taken at fre-
rvals for RIA. The hormone was
red at a dose of 1 mg in saline
then 6 hr later 1 mg in a gelatin
solution.

levels in sera of pregnant individ-
oups of timed pregnant mice
River CD-1), rats (Charles River
guinea pigs (Charles River Har-
re bled at frequent intervals throughout pregnancy and after parturition, and relaxin levels were determined by RIA.

RIA relaxin was also determined in blood samples obtained from late pregnant dogs, java and rhesus monkeys, baboons, and human beings. Control blood samples were drawn from nonpregnant individuals and males of the above species.

Results. Addition of 100 μl of male rat serum to each sample tube did not significantly alter the linear regression of the standard curve for porcine relaxin prepared in PBS-1% egg albumin (Fig. 1). Similarly, pooled plasma obtained from ovariectomized (ovx) women did not affect the standard curve obtained with porcine relaxin. Incubation of porcine relaxin in ovx human plasma at 37° for 24 hr did not influence the reactivity of the hormone with the antiserum (Fig. 2).

Following im injection of a saline solution of 1 mg of purified porcine relaxin into a female baboon, the serum concentration of RIA relaxin rose to a peak at 2.5 hr and then declined by 4.5 hr (Fig. 3). A second im injection of 1 mg of porcine relaxin was given in a 20% gelatin retardant vehicle 6 hr after the initial injection. A much smaller but protracted increase in serum RIA relaxin was observed for the duration of the experiment (Fig. 3).

RIA relaxin was first detected in serum on Day 9 of pregnancy in rats (Fig. 4). The

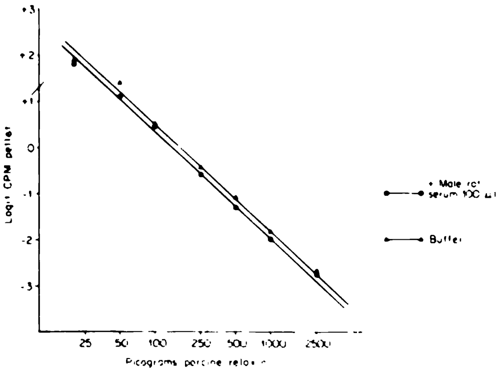

FIG. 1. Porcine relaxin standard curves in buffer or in buffer containing 100 μl of male rat serum. The linear regression for the two curves is as follows. Correlation coefficient: buffer = −0.9989; buffer + male serum = −0.9984. Slope: buffer = −1.0213; buffer + male serum = −1.0273. Logit intercept: buffer = 5.2570; buffer + male serum = 5.1382.

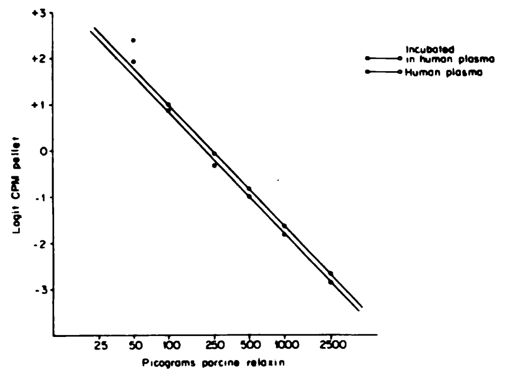

FIG. 2. Porcine relaxin standard curves. Open circles indicate samples which were incubated at 37° for 24 hr in human plasma. Closed circles are samples which were added to human plasma just prior to assay.

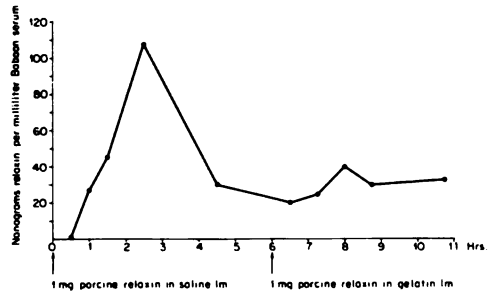

FIG. 3. RIA relaxin in serum of a baboon which received im injections of porcine relaxin in saline solution (time 0) or 20% gelatin (6 hr). Values greater than 20 ng/ml are significantly different from 0.

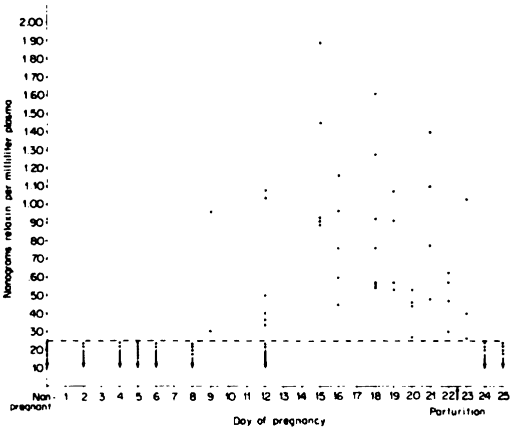

FIG. 4. RIA relaxin levels in sera of pregnant rats. Dotted line indicates sensitivity of the assay. Arrows indicate "less than."

levels of immunoreactive hormone increased until Day 15 of pregnancy and remained elevated until term, whereafter they fell to unmeasurable levels by 2 days postpartum (Fig. 4). A similar pattern of RIA relaxin was observed in sera of pregnant and postpartum mice (Fig. 5).

In guinea pigs, serum RIA relaxin rose gradually throughout the course of gestation and was still elevated on the day following parturition (Fig. 6).

Serum samples taken from a pregnant bitch 4 days prior to whelping and 1 month postpartum were assayed using serum from a male dog as control. A level of RIA relaxin equivalent to 2.4 ng/ml of serum was detected in pregnancy. No RIA relaxin could be detected in the postpartum sample.

Plasma samples taken from 19 women in the third trimester of pregnancy were assayed at 2 volumes each and calculated using standard curves containing equivalent volumes of pooled human male plasma.

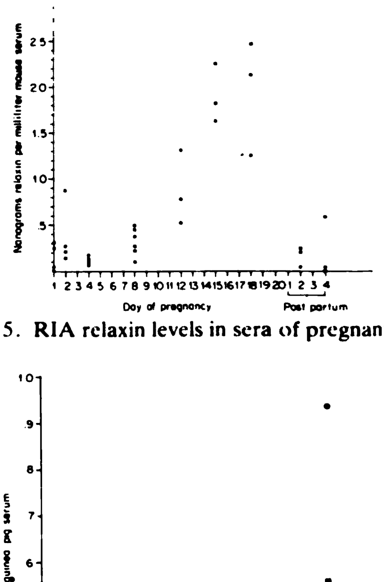

FIG. 5. RIA relaxin levels in sera of pregnant mice.

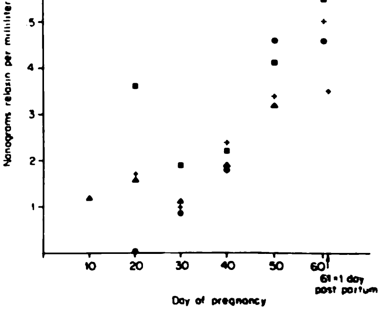

FIG. 6. RIA relaxin levels in sera of pregnant guinea pigs.

one crossreacting with the antibody
alent to 0.41 ± 0.03 ng of porcine
n/ml plasma was detected in these hu-
samples. Relaxin levels in pregnant in-
als of other primate species were as
s: rhesus monkey (Day 83) 0.27 ng/
va monkey (Day 76) 0.23 ng/ml; ba-
(five individuals, Days 27-147) 0.27
2 ng/ml.

cussion. Control serum obtained from
or castrated individuals was added to
ubes containing the porcine relaxin
ards to compensate for nonspecific ef-
of the volume of serum used to assay
wns. This procedure yielded a linear
nship between the volume of serum
d and the amount of relaxin detected.
ubation of porcine relaxin standards in
n plasma did not destroy the immuno-
vity. Thus, human plasma does not
r to have "relaxinase" activity.

en porcine relaxin in saline solution
jected into a baboon the serum level
rapidly to a peak at 2.5 hr and then
ed by the time the next blood sample
btained at 4.5 hr. This observation
sts that the hormone was rapidly ab-
d, metabolized, and/or excreted.
the relaxin was injected in a gelatin
e, serum levels of RIA relaxin rose
more slowly, suggesting a slower ab-
on from the site of injection.

relaxin was first detectable in serum
e ninth day of pregnancy in the rat.
vels rose between the twelfth and fif-
day of gestation and remained ele-
until after parturition. Anderson *et al.*
ported a similar increase in ovarian
n (bioassay) between Days 14 and 20
gnancy in rats. The rise in serum and
n relaxin thus immediately precedes
st detectable increase in cervical dilat-
on Day 13 of pregnancy in rats (8).
al dilatability then increases linearly
parturition, whereupon the cervix
up dramatically the first 2 days post-
n (8). Similarly in mice, serum RIA
levels rose precipitously between the
h and fifteenth days of gestation and
ed elevated until after parturition. In
the cervix progressively softens from
5 until term while interpubic ligament
tion proceeds from about Day 16 until
9). These data support the view that relaxin is secreted by the rodent ovary during the last third of pregnancy and is responsible for the changes observed in the connective tissue of the pubic symphysis and uterine cervix. Likewise in guinea pigs, which have a much longer gestation period, serum RIA relaxin was observed to increase from about Days 40 to 60 of pregnancy. Many workers have described the remarkable "relaxation" of the pelvic girdle which occurs during the last third of pregnancy in this species (see 10). In addition, Porter (11) has suggested that relaxin rather than progesterone may be the major myometrical regulating factor in the guinea pig. The various pregnancy-associated changes can be duplicated in nonpregnant rodents by injected porcine relaxin (8-11). Thus when past and present data are considered together, they strongly suggest that the serum factor identified by RIA in the present study is the physiologically active hormone, relaxin.

The relaxin levels measured in serum of pregnant animals by the present RIA reflect cross-reactions of their hormones with an antibody to porcine relaxin. The cross-reactions of pregnant rats and mice were equivalent to 1-2 ng of porcine relaxin/ml serum. The levels found in pregnant guinea pigs were less than 1 ng/ml. The pregnant dog serum was equivalent to 2-3 ng/ml in comparison to 0.2 to 0.3 ng/ml detected in pregnant non-human primates. In pregnant human beings RIA relaxin levels were about 0.4 ng/ml. These apparent species differences in absolute levels of RIA relaxin may actually be due to differences in structure rather than number of the various relaxin molecules. Those structures which are most similar to porcine relaxin would presumably cross-react more strongly with the anti-porcine relaxin antibody than those structures which differ significantly from the homologous hormone. In order to prove this point it would be necessary to isolate relaxin from tissues of the various species in sufficient mass to permit molecular weight determination and the quantitative preparation of solutions. However, we believe that the fluctuations seen during the course of pregnancy in any one species (e.g., rat) do reflect accurately the relative amounts of hormone being produced.

It is of interest that Sherwood *et al.* (1) were unable to detect RIA relaxin in pregnant guinea pig serum and found a difference in slope between "pregnant rat relaxin" and their porcine standards. These differences may be due to their use of different antisera and to their failure to add control sera of the species at assay to the standard tubes.

Summary. We have investigated the application of a RIA for porcine relaxin to the assay of relaxinlike substances in the blood of various other mammalian species. The cross-reactivity between antiporcine relaxin antibody and the relaxinlike substances in the blood of other mammals during pregnancy was sufficiently high to permit the assay of 0.1-0.5 ml of serum or plasma samples. Nonspecific reactivity was controlled by adding similar volumes of serum or plasma obtained from ovariectomized female or intact male subjects to RIA tubes containing known porcine standards.

RIA relaxin levels rose markedly during the last third of pregnancy in rats, mice, and guinea pigs. RIA relaxin was also found in late pregnancy in dog, rhesus and java monkeys, and human beings. The apparent blood levels of hormone found in each species will depend upon their degree of cross-reactivity with the antiporcine relaxin antibody as well as upon their actual concentration. Thus, absolute blood level values should not be taken literally. However, the fluctuations in RIA relaxin observed during the course of gestation in any given species would appear to reflect accurately the relative blood concentrations of the hormone.

We thank the following for their invaluable help during the course of this work: Mr. Wilbur Sawyer and Mrs. Margaret Butler for skillful help in various phases of the work; Dr. R. P. Blye, Center for Population Research, NIH, for blood samples obtained from a relaxin-treated baboon; Dr. Wendell H. Niemann, New York University Medical School (LEMSIP), for blood samples obtained from pregnant baboons, java and rhesus monkeys; Dr. Christian Schwabe, Medical University of South Carolina, and Dr. Albert Segaloff, Alton Ochsner Medical Foundation, for human pregnancy serum samples.

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Effect of Partial Starvation and Glucagon Treatment on Intestinal Villus Morphology and Cell Migration (39378)

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importance of gastrointestinal hor-
in the functional physiology of ali-
on has long been recognized. The
tion that one of these hormones,
could stimulate incorporation of
cids in gastric and duodenal mucosa,
d the earlier suggestion that gastrin
es might influence processes related
rowth and maturation in the gut (1,
sequently, other trophic effects of
testinal hormones have been re-
(3-8).

ed blood levels of the pancreatic is-
hormone glucagon (9) have been
ed with augmented intestinal trans-
amino acids and sugars in diabetic
, partially starved (13), and gluca-
ited rats (14). Further, changes in
owel motility, absorptive function,
icture have been reported in a pa-
th tumor secreting enteroglucagon
gut-derived hormone with immuno-
id some functional identities with
tic glucagon. These observations
: possibility that prolonged exposure
nented glucagon serum concentra-
ght alter intestinal mucosal structure
is function. This hypothesis is exam-
this report with studies of jejunal
logy and cell migration kinetics in
with hyperglucagonemia produced
ited injections of the hormone or by
tarvation (9).

al preparation. Male Sprague-Dawley rats weighing between 150 and 200g were used uniformly throughout this study. Animals treated with glucagon were given ip injections of 50 μg of Lilly glucagon in diluent every 6 hr for varying periods of time. Control animals for glucagon studies received 0.05 ml of glucagon diluent alone (containing 0.2% phenol as a preservative) or 0.05 ml of normal saline. The last injection was always given 1 hr before autopsy. Throughout the period of glucagon treatment, control and experimental animals were allowed free access to food and water. In this study, the term "partial starvation" refers to the diet of rats that have been given 6 g of Rockland Complete Mouse-Rat Diet per day per rat instead of the normal food intake of control rats, 22 g per day. Food was given to the starved animals in the evening. Control animals were allowed free access to food and water until autopsy.

Glucagon assay. Blood for glucagon assay was drawn from ether-anesthetized animals by aortic catheterization. All blood was collected into chilled tubes containing 2000 KIU of Trasylol. The blood was allowed to clot, and serum separated under refrigeration was then stored frozen until assayed for glucagon. Serum glucagon concentrations were measured by a standard double-antibody radioimmunoassay (9). ^{125}Iodine-labeled glucagon with a specific activity of greater than 350-400 mCi/mg was purchased from Cambridge Nuclear. The assay sensitivity was better than 20 pg/ml. Antibody to porcine, insulin-free glucagon was produced in guinea pigs. The second antibody used was rabbit anti-guinea pig immunoglobulin. The antiserum was not wholly specific for pancreatic glucagon, as it reacted minimally with purified enteroglucagon (kindly supplied by L. Heding of Novo Industries). Standard curves obtained at the beginning and at the end of the assay were

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titute (operated by the University of Chi-
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rved.

identical. All determinations were performed in triplicate. Variance within the assay was less than 10%. Interassay variance was negligible.

Morphology and cell migration by autoradiography. After 7 days of semistarvation or 5 days of 6-hourly glucagon injections, animals were given ip injections of 100 μCi of [*methyl*-^{3}H]thymidine in a 1-ml volume of 0.9% saline. At 1.5, 8, 20, and 32 hr after the injection, animals were sacrificed, and a segment of proximal jejunum 1-cm long was removed 12-cm distal to the pyloric valve. This segment was placed serosal side down onto a piece of cardboard and immersed in 20 ml of buffered formalin for 2 days. The 10% buffered formalin was made by mixing 7 g of monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$), 17 g of anhydrous dibasic sodium phosphate ($NaHPO_4$), 160 ml of formaldehyde, and enough water to make 1600-ml total volume at a pH of 7.0. The tissue was then dehydrated, embedded in paraffin, cut so that each section was at least 50 μm apart, and the sections put onto glass slides. The slides were coded with random numbers and dipped in undiluted Kodak NTB-2 liquid emulsion, allowed to dry, placed in light-tight boxes with desiccant, and stored in uniform orientation in a refrigerator for 8 weeks. At the end of this period, the slides were developed in Kodak Dektol Developer for 5 min at 19° and washed in running water at the same temperature for 30 min. The sections were then stained with Nuclear Fast Red and cover-slipped.

The slides were examined in a Bausch and Lomb microscope that contained a wide-field eyepiece with a grid calibrated for measuring. The grid contained 400 square units, each unit having 0.05175-mm sides at low power. Measurements were made of villus height, crypt depth, and the distance on the villus covered by radioactive grains visualized over nuclei. All measurements were made at 10× magnification, mucosal side of the muscularis mucosae as a base line. Only villi were measured which were oriented so as to demonstrate a vertical section through the crypt; thus an unbroken line of cells could be followed from the base of the crypt to the tip of the villus. After all the slides had been examined and recorded, the numbering code was broken.

Data are presented as the sample mean plus or minus the standard deviation. Straight lines in graphs were drawn by the method of least squares (16). The letter "b" is used to denote the regression coefficient of each line. Regression coefficients were compared with Student's *t* test to establish significant differences between plotted lines.

Results. Production of exogenous hyperglucagonemia. To examine the effect of ip injections of 50 μg of glucagon on serum levels of the hormone, sera were assayed at various intervals after an injection. The results are shown in Fig. 1. In a previous study, control animals had mean serum glucagon levels of 77 pg/ml, under the conditions of this assay (9). After injection, serum glucagon levels were above the normal range for the first 2 hr, but thereafter values returned to the range of physiologic function.

Effects of partial starvation and glucagon treatment on intestinal villus morphology and cell migration. To examine whether prolonged exposure to elevated plasma concentrations of glucagon might exert a trophic effect on jejunal mucosa, measurements of villus height and rate of villus cell migration were determined in animals made hyperglucagonemic with repeated doses of glucagon or by partial starvation. Partial starvation for 7 days has previously been shown to be associated with marked hyperglucagonemia in the rat (9). After 7 days of partial starvation or 5 days of 6-hourly glucagon injections, the mean villus length in both experimental groups was found to be significantly reduced. The crypt-to-villus ratio was unal-

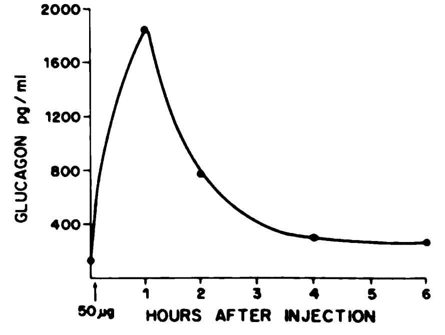

FIG. 1. The effect of exogenously administered glucagon on serum levels of the hormone. Fifty micrograms of glucagon were injected intraperitoneally.

tered by either treatment modality. These data are presented in Table I. Further, as shown in Fig. 2, the rate of cell migration was diminished with both partial starvation and glucagon treatment, but only glucagon therapy was found to reduce the rate of cell migration significantly ($p < 0.01$): (b = 7.8 $\times 10^{-3}$ mm/hr), compared to controls (b = 13.6 $\times 10^{-3}$ mm/hr).

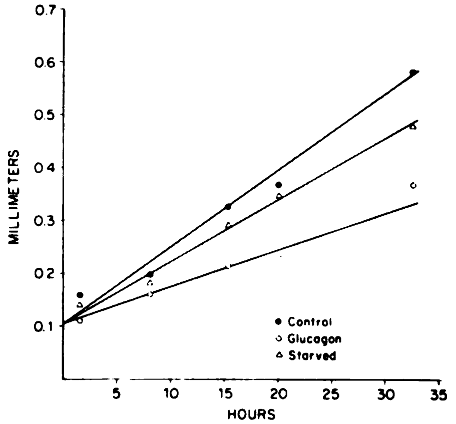

FIG. 2. The rate of cell migration in control, semistarved, and glucagon-treated rats. The rate was significantly decreased in glucagon-treated animals ($p < 0.01$). The difference in rate was not significant for semistarved animals.

Discussion. Active transport of amino acids and sugars has been shown to be augmented in two conditions associated with hyperglucagonemia in the rat, namely, partial starvation (9, 13) and diabetes (10–12). Recent studies reported from this laboratory have shown that chronic administration of glucagon to rats will also increase intestinal transport of amino acids and sugars (14). These functional alterations seen in association with hyperglucagonemia might be explained by a morphologic effect of the hormone on the intestinal mucosa.

It was the purpose of this study to determine whether the hyperglucagonemia previously shown to be associated with augmented intestinal transport might also be accompanied by altered intestinal morphology or cell migration kinetics. In both partially starved and glucagon-treated animals the mean villus length was found to be significantly reduced when compared to that of controls. The rate of cell migration was diminished by both partial starvation and glucagon treatment, but only the latter was found to reduce the rate of villus cell migration significantly compared to controls.

These observations in starved animals are in accord with those of other authors (18–21) who have also observed decreased villus height and a diminished rate of cell migration in starved animals.

Since villus height is decreased with both semistarvation and glucagon therapy, it might be argued that in both instances the animals probably had fewer transporting cells than control animals. Despite this, tissues from glucagon-treated (14) and semistarved animals (8, 12) have been shown to be able to absorb amino acids and sugars more effectively. It appears from Kinter's autoradiographs (17) that, as intestinal epithelial cells migrate upward on the villus and become more mature, they become progressively better able to transport. If this is true, then lengthening the residence time from crypt to villus tip might result in a population of more mature cells on the villus, and therefore increase the capacity for active transport of amino acids. A decreased rate of cell migration was observed in tissues from semistarved and glucagon-treated animals. Glucagon-treated animals appeared to have a slower rate of cellular migration than do semistarved animals, but the semistarved animals showed a greater stimulation of

TABLE I. THE EFFECT OF SEMISTARVATION AND GLUCAGON TREATMENT ON VILLUS LENGTH AND CRYPT DEPTH.

	Villus length in mm Mean ± SD	Crypt depth in mm Mean ± SD	Number of animals	Significance[a]
Control	0.75 ± 0.03	0.19 ± 0.02	15	–
Semistarved	0.66 ± 0.07	0.16 ± 0.02	14	$p < 0.001$
Glucagon-treated	0.61 ± 0.07	0.16 ± 0.02	14	$p < 0.001$

Note: Villus length and crypt depth were decreased by semistarvation and glucagon treatment.

[a] Significance figures apply to both villus and crypt measurements.

transport function. Therefore, it is not likely that an increased villus dwelling time alone could account for the increased transport seen in these two conditions.

The morphologic and cell migration data presented here and elsewhere (18, 19) raise some important questions about the relationship between intestinal cell migration and function. The technology is presently at hand to examine this problem further. An altered rate of intestinal cell migration and turnover can now be studied, and transport can be quantified, in any of a variety of situations (radiation, starvation, glucagon therapy, sprue, etc.). In this way it may become possible to examine the relationship between cell turnover, migration time, and functional parameters of the intestinal epithelium.

Summary. Male Sprague-Dawley rats were either treated with repeated ip injections of glucagon every 6 hr or partially starved. After 7 days of partial starvation or 5 days of glucagon injections, a time period shown previously to induce increased transport, all animals were sacrificed and a segment of jejunum was removed, fixed in formalin, sectioned, and dipped in Kodak NTB-2 liquid emulsion. After 8 weeks of exposure the autoradiographs were developed; assessments of villus height and crypt depth and measurements of length of the column of exposed grains were made in a calibrated microscope. The mean villus length in both semistarved and glucagon-treated groups was found to be significantly reduced ($p < 0.001$) when compared to control animals. The crypt-to-villus ratio was found to be unaltered by either treatment modality. The rate of cell migration was diminished by both partial starvation and glucagon treatment, but only glucagon therapy was found to cause a significant ($p < 0.01$) reduction in the rate of cell movement when compared to controls.

We are grateful to Dr. A. M. Lawrence for performing the glucagon assays.

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S OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 281-283 (1976)

ıcement of Insulin Secretion by Human Chorionic Somatomammotropin and Related Hormones (39379)

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esponse to a sustained glucose stimu-
ıe isolated perfused rat pancreas se-
insulin in a diphasic pattern charac-
l by an initial rapid secretory phase
ed by a decline in the rate of secretion
en a slowly rising rate of insulin secre-
1). Hypophysectomy alters this di-
response by producing a marked re-
n in the initial secretory response and
ıction that is not so striking in the
l or rising rate of insulin secretion (2).
nent of hypophysectomized rats for a
of 2 weeks with BGH[1] completely
s the second secretory phase to nor-
ıt has little effect upon the first secre-
hase (2). The amounts of BGH used
se experiments was 500 μg/day and
t injection was given the day prior to
on. Subsequent experiments (Bennett
ırry, unpublished data) indicated that
inimum effective dose of BGH for
ng the second secretory phase was
imately 200 μg/day. In this commu-
n we report the comparative effec-
s of HCS and related hormones
, PL-HGH, and OP) in restoring the
ıry response of the perfused pan-
rom hypophysectomized rats.

rimental. The perfusion technique
ıedium were those previously de-
l (1) except that the perfusion me-
contained Mg^{2+} at 2.4 mequiv/liter.
ımals used were male rats of the
ıe-Dawley strain weighing approxi-
300 g at the time of hypophysec-
Animals were hypophysectomized by
apharyngeal approach and were used
imately 4 weeks after hypophysectomy. The completeness of hypophysectomy was checked by inspection of the region of the sella using a binocular microscope. All hormones were given ip at a dose of 200 μg/day for 6 days with the last, or sixth, injection being given the morning of perfusion. Animals were fasted for approximately 20–24 hr prior to having their pancreases removed for perfusion. The isolation and purification of HCS (3, 4), HGH (5), and OP (6) have been described previously. PL-HGH was prepared by the procedure of Li and Graf (7), except that the final product was purified further by exclusion chromatography on Sephadex G-100 in 0.01 *M* NH_4HCO_3 of pH 8.2. Insulin was determined by the method of Grodsky and Forsham (8). The standard glucose stimulus (300 mg/dl) was started after a 10-min equilibration period and continued for 38 min.

Results and discussion. Histological studies of the pancreases were not done, but it is significant that the rats treated with HGH showed an average body weight gain of about 5 g/day as did those treated with PL-HGH. All rats treated with HCS and OP lost small amounts of body weight.

The pertinent data from these experiments are summarized in Table I. When one compares the data from the normal control animals with the hypophysectomized control animals, it is clear that as a result of hypophysectomy, the first secretory phase is strikingly reduced ($p < 0.001$) and the second secretory phase also shows a significant reduction ($p < 0.01$). The animals pretreated with HGH do not show significantly increased insulin release during the first phase compared to the hypophysectomized controls, but the first phase remains significantly depressed below the normal controls. On the other hand, the amount of insulin released during the second secretory phase

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reviations: BGH, bovine growth hormone;
ıman chorionic somatomammotropin; HGH,
ıituitary growth hormone; PL-HGH, plasmin-
human pituitary growth hormone; OP, ovine
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served.

TABLE I. *In Vitro* SECRETION OF INSULIN BY THE PANCREASES OF HYPOPHYSECTOMIZED RATS TREATED WITH HCS, HGH, PLASMIN-MODIFIED HGH, AND OVINE PROLACTIN.

	First secretory phase: Min 2-6			Second secretory phase: Min 25-40		
Experimental group[a]	Insulin release[b] (μg)	*P* vs normal control	*P* vs Hypx control	Insulin release[b] (μg)	*P* vs normal control	*P* vs Hypx control
Normal controls (4)	0.38 ± 0.03		<0.001	2.91 ± 0.19		<0.01
Hypx controls (7)	0.13 ± 0.02	<0.001		1.86 ± 0.17	<0.01	
Hypx + HGH (7)	0.23 ± 0.04	<0.05	NS	2.62 ± 0.29	NS	<0.05
Hypx PL-HGH (4)	0.32 ± 0.08	NS	NS	3.31 ± 0.44	NS	<0.02
Hypx + HCS (8)	0.26 ± 0.05	NS	<0.05	3.38 ± 0.35	NS	<0.01
Hypx + OP (4)	0.17 ± 0.03	<0.01	NS	2.41 ± 0.45	NS	NS

[a] Number of animals in parentheses.

[b] Mean ± SE.

is elevated and does not differ significantly from the amount secreted by the normal controls and is significantly higher than the amount secreted by the hypophysectomized controls.

PL-HGH produces an even more striking restoration of the ability of the pancreas to secrete insulin than does the intact HGH molecule. The total insulin released during the first phase is almost completely restored but there was large variation in the response and a small number of animals in the group. The total amount of insulin released during the second secretory phase is restored to that of the normal controls and is significantly elevated above the hypophysectomized controls.

The data from animals pretreated with HCS exhibit an increased insulin release during both the first and the second phase. In both instances the insulin release is significantly greater than that shown by the control hypophysectomized animals, and in neither case is it significantly different from that of the control normal animals. In striking contrast to the above findings is the lack of effect of OP on the first phase of insulin release. It is of interest that larger amounts of OP (5 mg/day) do produce some increased secretion in the first phase and also restore the second phase to normal (9).

The comparative effectiveness of HCS, HGH, and OP as recorded in Table I is somewhat surprising in view of their primary structures and biological properties. HCS is a potent lactogenic hormone but a weak growth-promoting agent (3, 10, 11) in comparison with HGH, whereas OP is the most potent lactogenic hormone and is practically free of growth-promoting activity in the rat (12). When the amino acid sequences of HCS (13), HGH (14), and OP (6) were compared, the homology between HGH and HCS amounted to 96% and the homology between HCS and OP was 60% (15).

The effect of HCS on insulin secretion is in accord with results reported by Malaisse *et al.* (16) who used pieces of pancreas in an *in vitro* system. We are unaware of any reports of effects of PL-HGH on insulin secretion but this material has been reported to be an active diabetogenic agent (17). It may also be noted that the growth-promoting and lactogenic activities of HGH are not altered by hydrolysis with plasmin (7).

There is good evidence that GH promotes insulin synthesis in response to a glucose stimulus (18) as measured by incorporation of radioactive leucine into insulin. On the other hand under the condition of these experiments, newly synthesized insulin is not necessarily released (19) nor does our experimental design permit such differentiations. What these experiments do demonstrate is that the ability of these four related hormones to produce bodily weight gain (presumably involving synthesis of somatic protein) cannot be correlated with their ability to promote insulin secretion in response to glucose. This ability does correlate with a high degree of homology in their amino acid sequences.

Summary. Hypophysectomized rats were treated for 6 days with 200 μg per day of either human chorionic somatomammotropin, human pituitary growth hormone, plas-

min-modified human pituitary growth hormone, or ovine prolactin. All hormone preparations except ovine prolactin enhanced the ability of the pancreases of hypophysectomized rats to secrete insulin in the isolated pancreas perfusion system.

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Radioimmunoassay of Procollagen in Serum of Patients with Paget's Disease of Bone (39380)

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Introduced by M. E. Lamm

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Tropocollagen is the triple helical molecule which aggregates to form the insoluble collagen fibers of the body. Tropocollagen is generated by the enzymatic conversion of procollagen, a soluble precursor molecule secreted into the extracellular space. The enzymatic conversion of procollagen entails the excision of nonhelical polypeptides from the ends of the molecule (1, for review). At least one of these excisions occurs *en bloc* to release a disulfide-linked, three chain fragment (2) from the carboxyterminus of the precursor (3-5). In contrast to triple helical tropocollagen, this excised fragment lacks hydroxyproline and hydroxylysine, has much less glycine and proline, and uniquely contains tryptophan and half-cystine residues (6).

The disulfide-linked carboxyterminal fragment of human procollagen has been isolated and purified and antibodies specific for it developed in rabbits (6, 7). A radioimmunoassay (RIA) for human procollagen has been developed which has demonstrated the antigen to be present in human sera (8). We now report that the RIA demonstrates abnormally high concentrations of procollagen protein in the sera of patients with Paget's disease of bone. Moreover, the serum procollagen concentrations decrease when the patients are treated with disodium etidronate (EHDP), a drug known to produce biochemical improvement in this disorder (9, 10).

Materials and methods. Patients. Thirty-one male and 19 female patients, ages 44-85, with Paget's disease of bone were included in this study. The diagnosis was based on characteristic roentgenographic findings, increased uptake of radioisotope into the affected bones, and elevated serum alkaline phosphatase values. None of the patients had received any therapy for Paget's disease for at least 3 months prior to the study. None of the patients had recent bone fractures or significant complicating illnesses. Sera were obtained prior to and after 3 months of therapy with 5-20 mg/kg/day of disodium etidronate (EHDP), supplied by Proctor and Gamble, Co., Cincinnati, Ohio. A detailed clinical analysis of this group of patients will be reported elsewhere. Informed consent for the study was given by all patients.

The patients were on a low gelatin diet when serum and urine samples were collected within the same 24-hr period. Serum alkaline phosphatase activity was measured by a modification of the thymolphthalein monophosphate method of Roy (11; normal range 13-41 units per liter). Total urinary hydroxyproline was measured by the method of Kivirikko *et al.* (12; normal range, 9-45 mg/24 hr).

Radioimmunoassay. Assays were performed as reported (8) except that radioactivity in the immune precipitates was measured in a gamma spectrometer. Sera were tested without knowing if they had been drawn before or after drug therapy. Purified, unlabeled antigen was used to generate a standard calibration curve for each group of sera tested. Percent displacements of radioactivity from immune precipitates were plotted against the log of the nanograms of standard antigen or microliters of test serum added. Concentrations of procollagen antigen in sera were calculated from points in the linear portion of the displacement curves (20-80% range; see Fig. 1). Independent assays of the same serum sample gave values which did not differ by more than 10%. The normal range for procollagen protein in adult serum is 5-15 μg/100 ml.

Results. Figure 1 shows the typical com-

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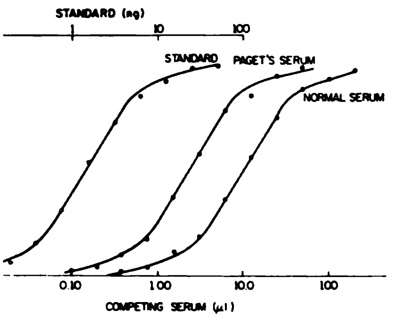

Radioimmunoassay: binding displacement
· standard antigen, a Paget's serum, and a
rum.

curves obtained when increasing
s of unlabeled standard antigen, a
ment Paget's serum, and a normal
rum, compete for antibody sites int
oimmunoassay. Complete displace-
radioactivity is achieved in all three
s and the slopes of the three curves
ıtical in the 20–80% displacement
'hese data support the view that nor-
l Paget's sera contain a molecule
cally identical to the purified procol-
andard.

les of serum from 44 patients were
ı the assay before and after treat-
ith EHDP, and these values were
ed with simultaneous determina-
urinary total hydroxyproline (Fig.
ıerum alkaline phosphatase activity
. All but four of the patients had
levels of procollagen before treat-
igs. 2a, 3a); the average value for
p was 52 µg/100 ml, a greater than
d elevation above normal. Figures 2
ıow that prior to treatment the in-
n urinary hydroxyproline and serum
phosphatase activity were generally
ıte with the increases in serum pro-
.

evated serum procollagen concen-
decreased significantly after treat-
th EHDP, and they returned to the
'ange in 33 of the 40 patients. The
procollagen concentration calcu-
these 40 patients receiving the drug
g/100 ml. The decrease in procolla-
centration was accompanied by a
in urinary total hydroxyproline
, and 66% of the patients achieved
evels of both parameters. The drug lowered the serum alkaline phosphatase levels in all patients (Fig. 3b), but in only seven instances did the values return to the normal range. On the average, the drug caused the pretreatment values for procollagen, urinary hydroxyproline, and alkaline phosphatase activity to decrease by 74, 79, and 67% respectively.

The average procollagen levels determined for six patients before and after administration of a placebo for 3 months were 58 and

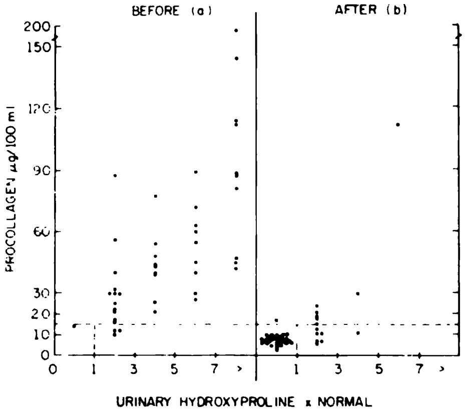

FIG. 2. Levels of serum procollagen and urinary total hydroxyproline before (a) and after (b) treatment with disodium etidronate. Hydroxyproline data plotted as multiples of the upper limit of the normal value. Results for 44 patients are given. Dotted lines define upper limits of normal for each variable.

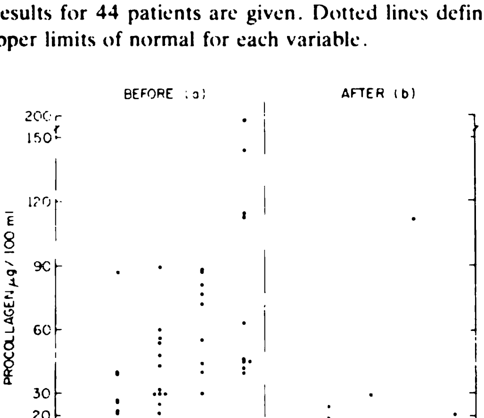

FIG. 3. Levels of serum procollagen and serum alkaline phosphatase before (a) and after (b) treatment with disodium etidronate. Alkaline phosphatase data plotted as multiples of the upper limit of the normal value. Same patient group as in Fig. 2. Dotted lines define upper limits of normal for each variable.

53 μg/100 ml, respectively (data not included in Figs. 2-3).

The RIA of normal serum was unaffected when EHDP was added to the assay tubes in concentrations greatly in excess of those occurring in the sera of patients taking the drug. We conclude that the levels of EHDP in the serum of treated patients did not interfere in the assay to produce artefactually low procollagen values.

Discussion. When the procollagen concentrations determined for 123 serum samples were matched with the record of treatment and the other clinical chemistries, the following correlations attested to the reliability and usefulness of the assay: (i) the procollagen concentrations in multiple pretreatment sera from a given patient were in good agreement, and 92% of such sera had significantly elevated concentrations of procollagen protein; (ii) significant decreases in procollagen concentrations were recorded for patients receiving EHDP, whereas the levels remained high in patients given a placebo; and (iii) the procollagen levels before and after treatment with EHDP were coordinate with the values for urinary total hydroxyproline and serum alkaline phosphatase activity.

Appropriate measurements demonstrate that bone resorption and synthesis are positively correlated in Paget's disease but that synthesis may lag behind resorption (13). Urinary hydroxyproline as measured in our study includes contributions from both bone resorption and synthesis (14-16). We therefore cannot decide from these data whether the procollagen levels of serum are only a function of collagen synthesis, or reflect collagen resorption as well. Although the metabolism of procollagen is by no means completely defined, we favor the view that neither the intact precursor nor the antigenic fragment excised from it become structural components of the insoluble matrix of bone. By this assumption, resorption of the matrix of bone in Paget's disease would not increase the soluble pool of procollagen protein, and the concentration of the latter would be primarily a function of its rate of synthesis. Studies are in progress to clarify this point. Contributions from tissues other than bone to the elevated procollagen levels in Paget's disease, while unlikely, cannot be excluded. However, the data as reported show that the RIA for procollagen in serum reliably reflects the changes in collagen metabolism occurring in Paget's disease of bone. We anticipate that the assay may be of value in other disorders affecting collagenous tissues.

Summary. A new radioimmunoassay for human procollagen showed that the sera of 46 of 50 untreated patients with Paget's disease of bone contained increased concentrations of procollagen protein as compared to normal adults. After therapy with disodium etidronate, all the elevated serum procollagen concentrations decreased significantly, falling to normal levels in 33 of 40 patients. The procollagen levels before and after treatment were coordinate with the values for urinary total hydroxyproline and serum alkaline phosphatase activity. The data show that the radioimmunoassay for procollagen is a dependable and useful adjunct to the study of Paget's disease of bone.

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Curie	Ci	molar (concentration)	*M*
degree Celsius (Centigrade)	-°	mole	spell out
degree Fahrenheit	°F	molecular weight	mol wt
diameter	dia	nanogram	ng
gram	g	nanometer	nm
hour	hr	normal (concentration)	*N*
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inside diameter	i.d.	ounce	oz
intramuscular	im	outside diameter	o.d.
intraperitoneal	ip	parts per million	ppm
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kilogram	kg	revolutions per minute	rpm
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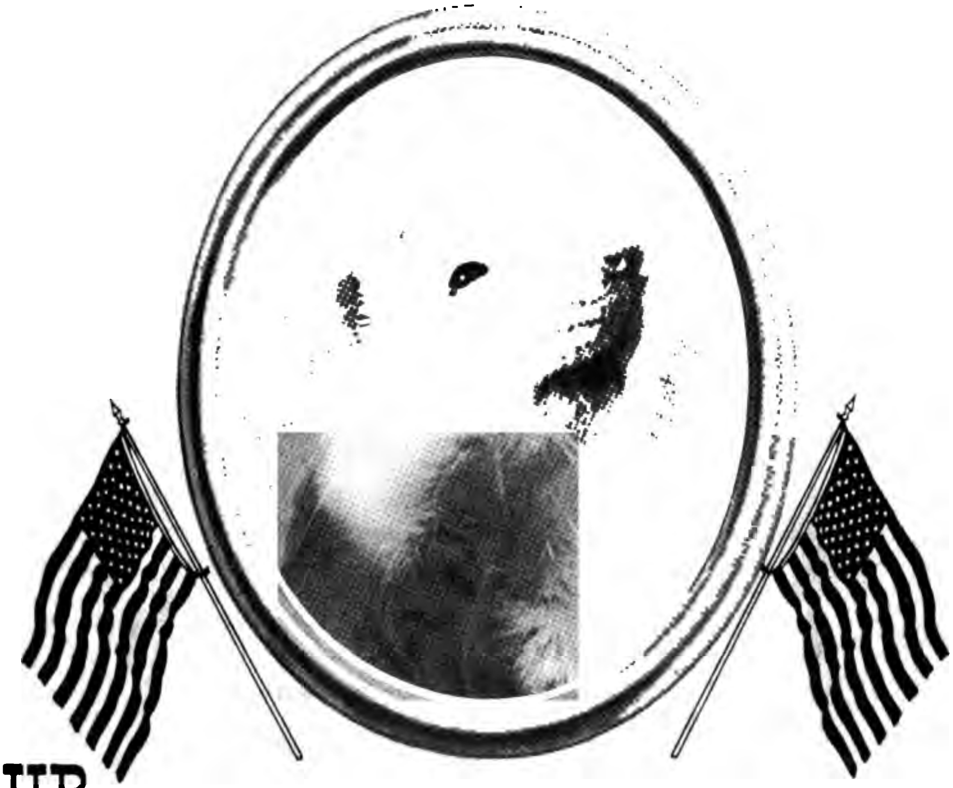

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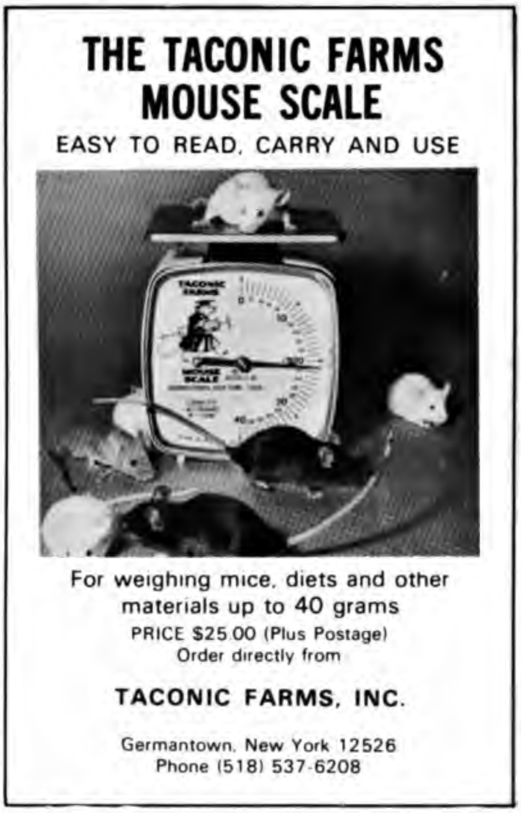

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NGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 289-291 (1976)

ngococcal Group C Subgroup Determinant Detected by Immunofluorescence[1]

(39381)

MICHAEL A. APICELLA AND HARRY A. FELDMAN

vision of Infectious Diseases, State University of New York at Buffalo, Buffalo, New York 14215; and
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Syracuse, New York 13210

ing an hemagglutination inhibition
od (HAI) and isolated capsular anti-
a subgroup determinant, C_{1+}, was
›nstrated in the polysaccharides of 26
) Group C meningococcal strains (1).
ıaccharides containing the subgroup de-
.nant were resistant to digestion by
ıminidase, 0.1 unit per milligram of
en, whereas those which lacked the de-
ınant, designated C_{1-}, were rapidly de-
ed by the same concentration of en-
. In this report is described an indirect
ınofluorescent antibody test system
) specific for the subgroup determinant
permitting the separation of intact
p C meningococci into C_{1+} and C_{1-}
'oups.

ıterials and methods. Cultures. Two col-
›ns of Group C meningococcal strains
studied with the IFA subgroup system:
0 strains previously studied with the
method (1), and (ii) 196 strains from
:ollection of one author (HAF). The
· were categorized only by IFA. The
ɔiazine sensitivity patterns of 121 of
.96 strains were determined (2). All
ıs were preserved lyophilized in Muel-
Iinton broth or in defibrinated rabbit
l at −70°.

ɪt preparation. Cultures were reconsti-
on tryptose blood agar plates and in-
:ed overnight at 35° under 5% CO_2.
:ria were removed from the agar me-
with loops and washed twice in phos-
:-buffered saline, pH 7.2 (PBS). They
resuspended in the same diluent at an optical density of 0.20 at 660 nm and approximately 10 lambda of each suspension were placed on glass slides, spread, and air-dried. All were studied on the day that the slides were prepared.

Subgroup test. Antiserum 20 produced in rabbits by immunization with a C_{1+} polysaccharide (3) and previously shown to contain antibodies to the subgroup determinant (1), was used as the unconjugated serum. This antiserum had been exhaustively absorbed with mouse liver powder and Group C meningococcal strains lacking the subgroup determinant by HAI. A sheep-anti-rabbit immunoglobulin antiserum was conjugated with fluorescein by the method of Clark and Sheppard (4); its *f/p* ratio was between 3 and 4. Slides were read on an American Optical series 10 Microstar microscope with a Fluorolume illuminator using 695 (Corning 5113) excitor and Schott OG-1 barrier filters. Positive and negative fluorescent staining were judged independently be two observers. HAI tests specific for Group C and subgroup determinants and neuraminidase digestions were performed as previously described (1). Capsular antigens for use as inhibitors of this system were prepared by the method of Robinson and Apicella (3).

Results. Initial studies with the subgroup IFA system were performed on the 20 Group C meningococcal strains which previously had been analyzed for the subgroup determinant by the HAI method. The 16 cultures which were C_{1+} in this system also were positive by IFA, while the four C_{1-} strains were negative by IFA. Among the second collection of 196 Group C meningococcal strains, 143 (73%) were IFA positive and 48 (24.5%) were negative. The remaining 5 (2.5%) cultures were both subgroup negative and positive. After replating, 10 single colonies were selected

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ıtional Institute of Allergy and Infectious Dis-
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:ch Career Development Award No. 5-K04-AI-
·02.

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from each for study by IFA. This time all were either subgroup positive or negative and not mixed as their parent cultures had been. In addition, Group C polysaccharides isolated from negative and positive colonies from the mixed cultures had the C_{1+} determinant only on the IFA positive subculture and not on the IFA negative one.

Further confirmation of the reliability of the IFA method was obtained from the following experiment. Polysaccharides isolated from 5 Group C, C_{1+} and 5 C_{1-} strains were analyzed for the subgroup determinant by HAI and their susceptibilities to neuraminidase determined. All strains identified as C_{1+} by IFA contained the subgroup determinant by HAI and were resistant to digestion by neuraminidase while the 5 C_{1-} strains lacked the determinant and were readily digested by the enzyme (Table 1).

The distribution of C_{1+} and C_{1-} strains appears to be unrelated to geography. Among 147 isolates from civilian and military sources in the continental United States, 110 (75%) were C_{1+} and 37 (25%) were C_{1-}. Of an additional 41 strains from five foreign countries on four continents, 32 (78%) were C_{1+} and 9 (22%) were C_{1-}.

Among the 196 strains, 15 were isolated from the nasopharynges of members of seven families with one or more cases. In these seven families, both case and carrier cultures were C_{1+} except for one family with two cases. The isolate from one child was C_{1+} and from the other was C_{1-}. In three other groupings of two cases each (apparently hospital-related), two pairs were both C_{1-} and the third was C_{1+}. All of these family and hospital groupings were of civilian origin. Multiple isolates from military cases and carriers were C_{1+} as were the carriers in a population of "normal" families (5).

TABLE I. NEURAMINIDASE SENSITIVITIES OF POLYSACCHARIDES OF MENINGOCOCCAL GROUP C SUBGROUPS DETECTED BY IFA AND HAI TESTS.

	Subgroup		Serogroup C (HAI)[a]	
Culture number	IFA	HAI (μg/ml)	Before (μg/ml)	After (μg/ml)
88	+	8.0	16.0	4.0
215	+	4.0	4.0	8.0
252	+	8.0	16.0	16.0
54	+	2.0	1.0	2.0
78	+	16.0	4.0	4.0
101	−	>1000[b]	16.0	>1000
194	−	>1000	4.0	>1000
125	−	>1000	2.0	>1000
144	−	>1000	8.0	>1000
53	−	>1000	16.0	>1000

[a] Before and after the digestion of 1 mg of polysaccharide with 0.1 unit neuraminidase for 16 hr.

[b] No inhibition at 1000 μg/ml.

TABLE II. COMPARATIVE FREQUENCIES OF SUBGROUP DETERMINANT IN STRAINS ISOLATED FROM CASES AND CARRIERS.

		Cases[a]		Carriers[b]	
C_1 subgroup	Sample number	Number	%	Number	%
+	132	97	73	35	27
−	34	31	91	3	9
Totals	166	128	77	38	23

[a] Spinal fluid, blood, joint, or eye.

[b] Nasopharynx, throat, sputum, or cervix. ($\chi^2 = 4.795, p = 0.05 > p > 0.025$.)

The sites of origin of study strains were available from 164 different individuals (Table II). C_{1-} strains were more often associated with clinical disease than were C_{1+} strains ($\chi^2 = 4.795, p = 0.05 > p > 0.025$). No statistically significant differences were found between susceptibility to sulfadiazine and subgroup type in 121 case strains ($\chi^2 = 2.207, p = 0.25 > p > 0.10$).

Discussion. Two systems for separating Group C meningococci into different subgroups have been reported. Gold and Wyle, using a bactericidal antibody assay directed against noncapsular cell wall factors, defined six serotypes. There seemed to be an association between increased attack rates (virulence) and resistance to sulfadiazine for serotype 2 (6). Counts *et al.* (7) described a typing system for Group B and C meningococci based upon the action of meningocidins. They identified 11 types with the majority being of type 1.

The subgrouping system described in this report utilizes IFA to identify a subgroup determinant present on the C polysaccharide. The results obtained with this system indicate that this determinant geographically is widely distributed. The presence or absence of the determinant and the strain's sulfadiazine sensitivity characteristics are unrelated. Our experience suggests that C_{1-} strains are associated with clinical disease to ıificantly greater degree than are those +. Whether this is related to virulence flects a sampling error remains to be mined.

e IFA method for subgroup detection pid and may be useful epidemiologi- . For the latter, as described herein, it be of value in defining potential menin- ccal contact clusters. Thus, application subgroup IFA procedure may help to ify the route of transmission of Group ningococci in outbreaks and in popula- carrier surveys. It also may have some in the selection of strains for vaccine ıction.

nmary. The presence or absence of a ngococcal Group C subgroup antigen can be detected by immunofluores- as readily as by a previously reported gglutination-inhibition method. When ared in strains isolated from cases and rs, subgroup negative (C_{1-}) strains d to occur more often in cases. The subgroup antigen is widely distributed geographically. The characterization of meningococci by its presence or absence may be of value in epidemiological studies.

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Effect of Fasting and Ileal Resection on the Concentration of Deoxycholic Acid in Rat Portal Blood[1] (39382)

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Despite the development of interest in the enterohepatic circulation of bile acids, little is known about their composition in portal blood. Most information has come from studies on rats (1-3) in whom cholic acid is the predominant bile acid. The amounts of other bile acids appear to vary considerably, there being a 30-fold difference in concentration of deoxycholic acid in studies (2) and (3). Although this could be partly explained by the different strains of animals used, it is also possible that feeding may have had an effect.

This study has been designed to investigate the effect of starvation on the composition and concentration of portal blood bile acids. Since the distal small intestine is the main site of active transport of bile acids (4), the effect of small bowel surgery on the "secondary" bile acids formed by bacteria in the gut has been examined.

Methods. Male and female Sprague-Dawley rats (250-300 g) were used. When required, portal blood (2-3 ml) was withdrawn from the superior mesenteric vein after ligation of the portal vein under ether anesthesia.

In the study of the effect of fasting, polyethylene cannulas (PP20, Portex Ltd., Kent, U.K.) were introduced into the splenic vein [similar to method A of Gallo-Torres and Ludorf (5)] and into the bile duct under pentobarbitone anesthesia immediately prior to the study. Purified [24-^{14}C]deoxycholic acid (52 μCi/μmole. Radiochemical Centre, Amersham, U.K.) (containing less than 0.4% as cholic acid) was then administered by two routes to rats which were either fasted (20 hr) or allowed to feed *ad libitum*. In the first experiment, 12 nmole were infused in isotonic saline (53 μl/min) over a 30-min period into the superior mesenteric vein. Bile was collected for 5-min periods during the infusion and then for a further 10 min. In the second experiment, a needle was positioned in the duodenum via the pylorus and [24-^{14}C]deoxycholic acid (24 nmole, 0.5 μCi, in saline) was injected as a bolus. Bile was collected for 5 hr, the animals being maintained under light pentobarbitone anesthesia.

In the study of intestinal resection animals were operated on under ether anesthesia. A laparotomy was performed in control animals and their intestines were divided and resutured. Distal ileal resections (20 and 50 cm) were performed 1 cm from the ileal caecal valve, end-to-end anastomosis being used to restore intestinal continuity. Animals were allowed to feed *ad libitum* on a standard laboratory diet postoperatively and portal blood was sampled 7 days later, between 9:00 and 10:00 AM. Control animals gained weight (mean 11%) over the 7-day period whereas animals in the experimental groups all lost weight: 20-cm ileal resection (5%), 50-cm ileal resection (9%), and right hemicolectomy combined with 20-cm ileal resection (22%).

Isolation of bile acids. Bile acids in serum (0.2-1.0 ml) and bile (50-100 μl) were isolated by liquid solid extraction using the resin XAD-7 (Rohm & Haas Ltd., Croydon, Surrey, U.K.) in a similar manner to that described for XAD-2 (6) except that 0.004 *M* sodium hydroxide was used to wash the resin since water alone caused elution of bile acids.

Analysis of biles. The bile acids were separated by tlc before and after hydrolysis with cholyl glycine hydrolase (7). The solvent systems used were chloroform:methanol:acetic acid:water (65:20:10:5, v/v) (8) for the conjugated bile acids, and 2,2,4-trimethylpentane:di-isopropyl ether:acetic

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[1] Supported by a grant from the Medical Research Council.

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pan-2-ol (2:1:1:0.4, v/v) for the
gated bile acids. The bile acids were
by spraying with iodine which was
blimed. The marked zones were
into scintillation vials and 0.5 ml of
ol was added to elute the bile acids
d by 10 ml of a scintillation cocktail
mes of toluene, containing 2.5 di-
xazole 4 g/liter and 1,4 di-2-(5
xazolyl) benzene 0.05 g/liter, and 1
Triton X-100]. The positions of the
al bile acids were confirmed by
g a separate track with 1 *M* sulphuric
d heating to 120° for 15 min.

ysis of sera. A portion of the initial
was acidified (pH 1) with 6 *M* hy-
ric acid and extracted three times
ethyl ether (3 volumes) in order to
e the unconjugated bile acids. The
ile acids (conjugated and unconju-
were examined by deconjugation of a
portion of the initial extract with
lycine hydrolase (7). The resulting
gated bile acids were converted to
ethyl esters by treatment overnight
ethanol containing acetyl chloride
v/v). After addition of internal
d, 5α-cholestane, the methyl esters
eated with pyridine, hexamethyldi-
, and trimethylchlorosilane (9:3:2,
) to form their *O*-trimethylsilyl ether
ives. These were purified after dilu-
th *n*-hexane (1 ml) by washing with
1 ml) to remove pyridine and excess
g reagent. Gas chromatography was
out in a 5-ft × 1/4-in. O.D. glass
containing 1% Hieff-8BP on Gas
Q (100–120 mesh) (Applied Science
tories, State College, Pennsylvania)
with a nitrogen flow rate of 60 ml/
etection was by flame ionization and
ative data obtained by comparison of
eight ratio to 5α-cholestane with
ds. Recoveries through this proce-
re checked by the addition of known
s of glycine and taurine conjugates
bile acid (10 and 50 nmole) to bile
e serum for every analysis and an
riate correction made. They were
to vary between 75–85% for di- and
xy bile acid conjugates and 55–65%
cholyl conjugates. Recovery of the
gated bile acids present in serum
was greater than 95%. The sum of the individual bile acid concentrations has been reported as the total bile acid concentration.

Bile acids isolated from the serum from portal blood were dissolved in chloroform:methanol (1:1, v/v) and analyzed by tlc using the system 2-methylbutyl acetate: propanoic acid:propan-1-ol:water (4:3:2:1, v/v) (10) in order to examine the state of conjugation. Bile acids were located by spraying with 1 *M* sulphuric acid heating at 120° for 15 min and viewing the fluorescence generated by exposure to 354-nm light.

Liver histology was examined by light microscopy. The pathologist had no previous knowledge of the type of operation that had been performed.

Results. Fasting (20 hr) led to no significant change in the total concentration of serum bile acids in portal blood ($150.8 \pm 20.4 \rightarrow 193.6 \pm 24.9\ \mu M$) in male animals. There was, however, a sharp rise in the deoxycholic acid concentration ($7.7 \pm 2.1 \rightarrow 26.9 \pm 4.0\ \mu M$, $p < 0.005$): this increase was reflected mainly in changes in the concentration of conjugated deoxycholic acid (Table 1).

To ascertain whether the increase in deoxycholic acid was due to a failure in 7α-hydroxylation by the liver [24-^{14}C]deoxycholic acid was infused into the superior mesenteric vein and bile collected. A total of 85% of the administered isotope was recovered in the bile of both fed and fasted animals within the period (40 min) of the experiment. Additional experiments were performed in which the isotope was injected into the upper small intestine. Recoveries of greater than 80% of the administered radioactivity in the bile occurred within 5 hr.

Analysis of the biles revealed no difference in the percentage of isotope hydroxylated to cholic acid between fed and fasted animals in the infusion study (Table 2). The values observed (2.5–8.9%) were much less than those reported previously (30–40%) (11, 12) when the isotope was given orally, although Hoffman, Iser and Smallwood observed no conversion of deoxycholic acid to cholic acid when it was infused into the portal vein (13). In order to see whether this difference was due to the method of admin-

TABLE I. EFFECT OF FASTING (20 HR) ON THE CONCENTRATIONS (μM) OF SERUM BILE ACIDS IN MALE RAT PORTAL BLOOD.[a]

	Cholic acid		Deoxycholic acid	
Total	Conj.	Unconj.	Conj.	Unconj.
Fed (6)				
150.8 ± 20.4	102.7 ± 20.7	40.4 ± 6.7	4.0 ± 1.3	3.7 ± 0.9
Fasting (6)				
193.6 ± 24.9	132.3 ± 18.7	34.4 ± 8.6	17.8 ± 3.0	9.1 ± 1.4
			$P < 0.005$[b]	$P < 0.01$[b]

[a] Results given as mean ± standard error.

[b] Test of significance, unpaired Student's t test. Where no p value is given, the difference was not significant at the 5% level.

TABLE II. CONVERSION OF [24-^{14}C] DEOXYCHOLIC ACID TO CHOLIC ACID.[a]

Route of administration	Total biliary radioactivity in cholic acid (%)	
	Fed animals	Fasted animals
Portal vein (4)	4.9 (2.5-8.1)	4.8 (2.5-8.9)
Intestine (2)		
1 hr	7.4 (6.4-8.3)	4.1 (4.1-4.2)
1-5 hr	7.8 (6.6-9.0)	8.6 (7.1-10.5)

[a] Mean and range given.

istration [24-^{14}C]deoxycholic acid was injected into the duodenum, but again there was only a little 7α-hydroxylation to cholic acid (4.1% fasted and 7.4% fed): the proportion of labeled cholic acid did not change during the experiment (Table 2).

Effect of intestinal resection. Since fasting had a marked effect on the serum concentration of deoxycholic acid in portal blood it was decided not to starve other experimental animals before sampling blood in order to examine the effects of bowel surgery alone.

There was a wide scatter of results in control animals although the mean total serum bile acid concentration in portal blood (139 μM in male animals, 137 μM in female animals) was similar to that observed in the fasting study. Chenic acid was a prominent bile acid in female animals (mean 17 μM) but not in male animals. Traces of lithocholic acid (0.5 μM) were observed in female animals, but hyodeoxycholic acid and the α- and β-muricholic acids were not detected in any of the animals (limit 0.5 μM).

Resection of the ileum in male rats, both 20 and 50 cm, caused a significant fall in the mean concentration of total cholic acid (134 μM to 24 and 17 μM, respectively, $p < 0.001$) whereas that of deoxycholic acid rose (from 5.6 μM to 12.7 and 9.1 μM, respectively), mainly because of unconjugated deoxycholic acid (Table 3) although this rise was not significant. Similarly in female animals with a 20-cm resection there was a significant decrease in the mean total cholic acid concentration (115 to 45 μM, $p < 0.02$) and a rise in the total deoxycholic acid concentration (3.7 to 16 μM), although again not significantly. In addition the mean concentrations of total chenic acid[3] and lithocholic acid were increased from 17 to 42 μM and 0.5 to 6.5 μM, respectively. Consequently there was only a small decrease in the total serum bile acid concentration in portal blood (from 138 to 111 μM). Analysis by tlc showed that conjugated cholate in the portal blood from all the sera was present as cholyltaurine.

Male animals who had a right hemicolectomy in addition to an ileal resection also had reduction in the total bile acid concentration. However, deoxycholic acid was absent from the sera of portal blood in all animals except one. All these animals had diarrhea.

When the bile acid composition in portal blood was examined 6 months after surgery in a small series (three animals in each group), similar qualitative changes were obtained to those seen after 1 week. However, the concentration of all bile acids were higher than those observed in the younger animals. In controls the mean total bile acid concentration was 275 μM and in animals with ileal resection was 121 μM (20 cm) and 70 μM (50 cm). For deoxycholic acid the mean concentrations in animals with ileal

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[3] Chenic acid: abbreviated name for 3α,7α-dihydroxy-5β-cholan-24-oic acid.

	Sex	No. of rats	Total bile acid concentration	Total	Unconj. (%)	Total	Unconj. (%)	Total	Unconj. (%)	Total	Unconj. (%)
Control	M	9	139 ± 21.3	133 ± 20.6	28	5.7 ± 1.4	39	<0.5	–	<0.5	–
	F	4	137 ± 24.3	115 ± 20.4	10	3.7 ± 1.4	46	17.3 ± 4.1	8	0.5 ± 0.3	tr
Ileal resection											
20 cm	M	8	37 ± 6.5 $p < 0.001$[b]	24.4 ± 5.4 $p < 0.001$[b]	29	12.7 ± 3.6 NS[b]	71	<0.5	–	<0.5	–
50 cm	M	8	27 ± 6.2 $p < 0.001$[b]	17.4 ± 3.8 $p < 0.001$[b]	47	9.1 ± 3.5 NS[b]	75	<0.5	–	<0.5	–
20 cm	F	4	111 ± 19.0 NS[c]	46 ± 7.3 $p < 0.02$[c]	44	16.0 ± 3.9 NS[c]	50	42.0 ± 9.3 $p < 0.05$[c]	38	6.5 ± 2.8 NS[c]	35
Ileal resection (20 cm) and right hemicolectomy	M	7	52 ± 12.4 $p < 0.01$[b] NS[d]	49.8 ± 12.4 $p < 0.01$[b] NS[d]	16	[e]	–	<0.5	–	<0.5	–

[a] Mean ± standard error given.

[b] Unpaired Student's *t* test against male control animals.

[c] Unpaired Student's *t* test against female control animals.

[d] Unpaired Student's *t* test against male animals with 20-cm ileal resection.

[e] Deoxycholic acid was found in one animal only. NS, not significant.

resection were 27 *μM* (20 cm) and 10 *μM* (50 cm): whether this was due to increases in age and mean body weight is not known.

Histological examination of the rat liver showed only minor changes. One week after ileal resection slight fatty changes were observed in female rats. Similar observations were made in male rats 6 months after surgery.

Discussion. The concentrations of total bile acids in the serum of portal blood of control rats covered a wide range (66–273 *μM*) of the same order observed by Okishio and Nair (3): the very low values previously observed by Cronholm and Sjövall (2) were not encountered. The extended sampling period (up to 30 min) used by these investigators (2) compared with 30–40 sec in our study could have been responsible for this difference. All studies were unanimous in showing that cholic acid was the major bile acid present in portal blood. Qualitative tlc analysis showed that cholic acid was conjugated almost entirely with taurine, this being consistent with the known composition of rat biliary bile acids. Other bile acids were present in trace amounts in male animals, but only deoxycholic acid was in a concentration exceeding 0.5 *μM*. However, in female animals 10% of the total bile acids consisted of chenic acid which is consistent with a previous report of a sex difference in the chenic acid pool size in the rat. (14).

An overnight fast (20 hr) caused a marked increase in the concentration of deoxycholic acid in male animals from 8 to 27 *μM* which is in good agreement with the results of Cronholm and Sjövall (2) for fed animals and Okishio and Nair (3) for fasted animals. Since the increase was mainly due to deoxycholyltaurine it seemed unlikely that increased bacterial action caused by slowed intestinal transit could explain this effect, as it is generally accepted that bacterial deconjugation of bile acids precedes their dehydroxylation although evidence for direct dehydroxylation has been reported in man (15). It has been previously shown (11, 12) that deoxycholic acid is converted to cholic acid by hepatic 7α-hydroxylation so that the possibility has to be considered that fasting may have decreased the capacity of the liver for 7α-hydroxylation and thus increased the amount of circulating conjugated deoxycholic acid. However, in experiments in which isotopic deoxycholic acid was administered either via the superior mesenteric vein or into the small intestine, analysis of the biles revealed no difference in the amount of labeled cholic acid between fed and fasted animals. A decreased rate of enterohepatic cycling of bile acids is a further possible explanation, but it is difficult to reconcile with the fact that deoxycholic acid was in the conjugated form.

It has been concluded from these observations on fasting that, as that rat is mainly a nocturnal eater and does not normally fast for long periods, studies on bile acid metabolism should normally be made in fed animals. This procedure was accordingly followed in our investigations on ileal resection which demonstrated that short (20 cm) and long (50 cm) terminal ileal resection caused similar reductions in the total serum bile acid concentration in portal blood. This was largely due to a decrease in the concentration of cholyltaurine which is consistent with a previous report of a threefold fall in the cholyltaurine pool size following resection from 21 μmole/100 g body wt to 7.2 μmole/100 g body wt (16) and supports the view that the distal small intestine is necessary for its conservation.

Since taurine conjugates of each of the bile acids were present in the serum of portal blood this would suggest that significant absorption of these polar bile acids can occur in the absence of the terminal ileum *in vivo*, as conjugation is unlikely to occur in the intestine. The mechanism of their reabsorption is uncertain, although active transport has been reported in the third quarter of rat small intestine (17). In addition the rate of absorption of cholyltaurine from the jejunum following ileal resection has been shown *in vitro* to be twice that in controls (18), and that this is not just because of jejunal hyperplasia but reflects a greater ability to concentrate cholyltaurine against a concentration gradient (19).

The deoxycholic acid concentration was raised in both male and female animals following ileal resection, mainly in the unconjugated form, although the scatter of the data prevented a significant difference being observed. This would follow from an increased rate of production of deoxycholic acid by anaerobic bacteria caused by greater amounts of unabsorbed cholyltaurine entering the colon, and a greater intestinal conservation of the unconjugated over the conjugated forms by passive non-ionic diffusion (20). No explanation can be offered for the apparent increase in the concentration of chenic acid in female rats.

Although the combination of a right hemicolectomy with resection of the terminal ileum did not influence the concentration of cholyltaurine it resulted in the disappearance of deoxycholic acid from the portal blood. Despite the intestinal hurry observed in these animals not all bacterial activity towards bile acids was suppressed since some unconjugated cholic acid was present. In man a similar operation also results in a diminution in deoxycholic acid synthesis (21).

Bile acids have been shown to be hepatoxic when given in large doses in experimental studies (22–26). Since ileal resection resulted in a reduction in the total serum bile and concentration in portal blood and only moderate rises in the concentration of secondary bile acids, it was therefore not surprising that no histological damage was detected in the livers of these animals.

Summary. Bile acids in the serum of rat portal blood have been examined. Fasting (20 hr) caused a marked increase in the deoxycholic acid concentration, mainly of deoxycholyltaurine. Studies with [24-^{14}C]deoxycholic acid failed to show that this was due to decreased rehydroxylation by the liver. Resection of the terminal ileum caused a three- to fourfold reduction in the total bile acid concentration in male animals, cholyltaurine being most affected although it remained the predominant bile acid. In contrast the concentration of deoxycholic acid increased in half the animals. In female animals the concentrations of chenic acid and lithocholic acid also increased so that the total bile acid concentration only decreased slightly. Ileal resection combined with a right hemicolectomy caused the disappearance of deoxycholic acid.

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Incorporation of ^{14}C-Docosahexaenoic Acid into Lipids of Rats (39383)

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Docosahexaenoic acid (22:6n3), a member of the linolenate family, is found in membrane phospholipids of most mammalian cells. It occurs in surprisingly high concentrations in the phosphatidylethanolamines (PE) of human cerebral cortex (1), retinas of various species, including human, dog, pig, sheep, cattle, and rabbit (2), and bovine testes (3) as well as other specialized cells or organelles. Large proportions of 22:6n3 also appear in many lipids of fish. Docosahexaenoic acid cannot be totally synthesized in vertebrates so that it or its precursor, linolenic acid (18:3n3), must be obtained from the diet. Rainbow trout grow well with 22:6n3 or 18:3n3 as the only dietary unsaturated fatty acid (4), but linoleic acid (18:2n6), the most important essential fatty acid (EFA) for mammals, is of little value (5). A great decrease in 22:6n3 of tissue lipids occurred in rats fed a diet deficient in linolenic acid, but growth and reproduction remained normal (6). Despite its essentiality in trout, and its high concentrations in many vertebrate membrane phospholipids, nothing whatever is known about the specific function of 22:6n3.

Little is known about the incorporation of 22:6n3 into tissue lipids. Sinclair and Crawford (7) and Sinclair (8) administered ^{14}C-22:6n3 to rat pups and found considerable incorporation into brain and liver phospholipids after 22 hr. No investigation has been made of the incorporation of 22:6n3 into lipids at different intervals after administration, a test which would allow comparison of the behavior of 22:6n3 with that of other fatty acids. Therefore, we have measured the incorporation of 22:6n3 into rat tissue lipids at various times after administration. Normal rats were administered ^{14}C-docosahexaenoic acid prepared by biosynthesis from $^{14}C_1$-linolenic acid in EFA-deficient rats. Incorporation of the label into lipids of liver, heart, plasma, brain, and carcass was measured after intervals from 0.25 to 95 hr. We also investigated the distribution of radioactivity among liver lipid classes and within individual fatty acids of liver phosphatidylcholines (PC) and PE.

Preparation of ^{14}C-docosahexaenoic acid. ^{14}C-docosahexaenoic acid was isolated from liver phospholipids of EFA-deficient rats after administration of $^{14}C_1$-linolenic acid. Just after the birth of litters, mother rats (Sprague-Dawley, Horton's Laboratories, Los Gatos, Calif.) were given an EFA-deficient diet (9). Pups were fed this diet and raised to weights of 160-180 g (8-10 weeks of age). $^{14}C_1$-Linolenic acid (Dhom, North Hollywood, Calif., 52.5 mCi/mmole, stated to be >99% pure) was mixed with 20% rat plasma in saline. About 30μCi in 0.3 ml was administered (Metofane anesthesia, Pitman-Moore, Washington, Pa.) via the femoral vein. After 72 hr, the animal was anesthetized (sodium pentobarbital), bled by heart puncture, and the liver was removed and lyophilized.

Liver lipids were extracted with chloroform:methanol 2:1 (v/v), and phospholipids were isolated from the total lipid extract by column chromatography on silicic acid (Unisil, Clarkson Chemical Co., Williamsport, Pa.). Neutral lipids were eluted with chloroform, and phospholipids were collected in chloroform:methanol 1:1 and in pure methanol. Mixed phospholipids were converted to methyl esters in methanol acidified with H_2SO_4, under nitrogen in a capped tube for 3 hr or more at 80°.

Methyl esters were separated and isolated by gas-liquid chromatography (glc) with a model 810 F&M chromatograph equipped with a Packard Tri-Carb fraction collector (Model 830). The instrument was calibrated with a mixture containing weighed amounts of the methyl esters of palmitic (16:0), pal-

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(16:1n7), stearic (18:0), oleic), 18:2n6, 18:3n3, arachidonic), eicosapentaenoic (20:5n3), and acids (Hormel Institute, Austin, Methyl esters were collected in acked with glass wool which were n scintillation vials and counted with mixture (below). Their specific ac- were determined by use of ^{14}C-hep- oic acid as an internal standard (Nu- icago). An average liver contained mg of 22:6n3, of which two-thirds overed as purified methyl docosa- ate, sp act 8.0 × 10^5 dpm/mg. The docosahexaenoate peak contained of the radioactivity and 88% of the ith the remainder distributed among very small unidentified components eared both before and after 22:6n3 GLC trace. For administration to ethyl docosahexaenoate was con- o the free acid and dispersed in 20% na in saline.

oration of ^{14}C-docosahexaenoic o rat lipids. Male Sprague-Dawley orton's; about 175 g) were given a ified diet for 2 weeks as described 10), except that corn oil was substi- r cottonseed oil. They were anesthe- th Metofane, and ^{14}C-docosahexae- d in diluted serum was injected via oral vein. Two or more rats per time were killed after 0.25, 0.5, 1, 2, 4, 6, 24, 40, 48, 72, and 95 hr. The radioactivity varied from about 2 × n, for incorporation up to 24 hr, to × 10^5 dpm, for longer incorpora-

were anesthetized and blood was y heart puncture. Plasma was sepa- nd an aliquot was counted in Triton (below). Brain, liver, and heart ophilized and their lipids were ex- into chloroform:methanol, 2:1. The ler of the animal including intestinal (carcass) was digested with satu- OH in 95% ethanol. Aliquots of digests were neutralized with and lipid-soluble material was ex- into petroleum ether for measure- radioactivity.

tical procedures. Lipids in organic were taken to dryness under nitro- counted in 0.5%, 2,5-diphenyloxazole ("PPO," Research Products International, Grove Village, Ill.) in toluene ("toluene mixture") in a Beckman LS-100 liquid scintillation counter (81% efficiency).

Liver lipid extracts were fractionated into lysolecithins, sphingomyelins, phosphatidylcholines (PC), phosphatidylinositols + phosphatidylserines, phosphatidylethanolamines (PE), and neutral lipids, by the thin-layer chromatography (tlc) system of Skipski *et al.* (11). Duplicate samples were streaked on silica gel H (Merck) plates. Fractions from one side of the plate were transferred into tubes and were eluted with methanol for determination of lipid phosphorus (12). The corresponding bands were scraped directly into scintillation vials and were counted in Triton mixture (928 ml of toluene, 544 ml of Triton X-100 (Rohm and Haas) and 4 g of PPO) (61% efficiency).

Another separation of liver lipid extracts was made for glc analysis of methyl esters from PE and PC. The two phospholipids were eluted from silica gel by the method of Arvidson (13). PE and PC were converted to methyl esters, which were separated and collected by glc as described above. With the use of the internal standard, the sp act of each individual fatty acid was measured.

All solvents and chemicals were of reagent grade.

Results and discussion. Radioactivity in lipids of heart, plasma, or brain was less than 2% of the total recovered radioactivity. These lipids were not analyzed further. The degree of incorporation did not vary in any recognizable pattern from one time period to another in these tissues.

At all times, the greatest proportion of lipid radioactivity was recovered in carcass lipids. This is not surprising since 22:6n3 accounts for 3 to 4% of the total fatty acids in skeletal muscle (14), which in turn accounts for 45% of body mass in rats. Thus the carcass contains most of the body's 22:6n3. We did not analyze the radioactivity in individual fatty acids of carcass lipids because specific activities were very low.

Liver lipid contained 11% of the total recovered radioactivity after 0.25 hr of incorporation and by 4 to 8 hr, it contained about 30% of the radioactivity recovered in the whole animal.

In liver lipids, most of the activity was incorporated into fatty acids of PE and PC, with much smaller proportions incorporated into neutral lipids and other phospholipids. The percentages of liver radioactivity in PE and PC are shown in Fig. 1. At the beginning of incorporation, a large proportion, 60–70%, of radioactivity appeared in PE, with little in PC. As time progressed, the values converged, so that by about 16 hr, approximately 45% of liver total radioactivity was present in each fraction.

Not all of the radioactivity was located in the 22:6n3 moiety. After 0.25 and 0.5 hr of incorporation, about 55–60% of the radioactivity in PE was in 22:6n3, whereas in PC only 25% of the label was in 22:6n3 (Table I). The remainder of the radioactivity was found mainly in C-16 and C-18 chains. This indicates that part of the 22:6n3 was quickly metabolized and that the label was reincorporated into fatty acids. As time progressed, the proportion of radioactivity in 22:6n3 increased, so that by the end of the experiment, 22:6n3 accounted for 75–80% of the label in PE, and about 80–85% of the label in PC.

For at least the first 10 hr of incorporation, we found more radioactivity in PE than in PC. This prompt incorporation of 22:6n3 into PE and its delayed incorporation into PC is quite unlike the incorporation behavior of 16:0, 18:0, 18:1n9, 18:2n6, or 20:4n6, the other major fatty acids in rat liver phospholipids. For example, when 16:0 was administered intravenously to rats, PC had more than twice the radioactivity present in PE (15) at 10 min after the dose and at 20 min after the dose.

TABLE I. RADIOACTIVITY IN FATTY ACIDS OF RAT LIVER PHOSPHATIDYLETHANOLAMINES (PE) AND PHOSPHATIDYLCHOLINES (PC) AFTER ADMINISTRATION OF ^{14}C-DOCOSAHEXAENOIC ACID.[a]

Time (hr)	Number of rats	Percentage as 22:6n3 in phosphatidylethanolamines	Percentage as 22:6n3 in phosphatidylcholines
0.25	2	55	27
0.5	2	61	24
1	2	69	46
2	2	68	49
4	4	67	56
8	2	76	52
10	2	65	63
16	4	66	61
24	2	54	57
40	2	79	73
48	2	77	79
72	2	80	80
95	2	76	85

[a] Percentage of total radioactivity in fraction recovered as 22:6n3.

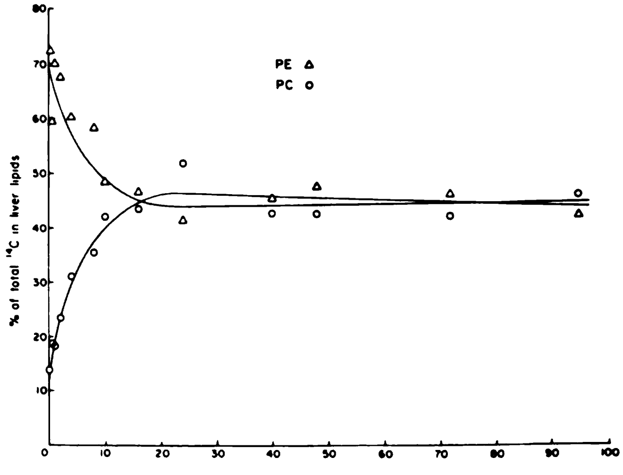

FIG. 1. Radioactivity in phosphatidylethanolamines (PE) and phosphatidylcholines (PC) of rat liver after administration of ^{14}C-docosahexaenoic acid. Percentage of total radioactivity recovered in liver total lipids. Each point represents the mean of two analyses for each of two animals, except for points at 4, 16, and 48 hr, which represent four animals. Agreement between values for replicate animals was usually within ±5% of the mean, with a maximum range of ±11%.

ad three times the amount of radioac-
that PE did (16). Similarly, 18:0 and
9 were incorporated in greater
nts into PC than into PE of rat liver
somes, at 1, 2, 4, and 24 hr after
istration of these fatty acids by stom-
ube (17) to rats deficient in EFA.
t and colleagues (18) administered
18:2n6, or 20:4n6 via the jugular vein
le Wistar rats, and more activity ap-
d in liver PC than in PE after 10, 30,
nd 180 min of incorporation.

selective incorporation of 22:6n3
iver PE is consistent with observations
in vitro with rat liver microsomal sys-
Kanoh and Ohno (19) showed that
1,2-^{14}C-ethanolamine was converted
ively into PE molecules that contain
3, an indication that the PE-synthesiz-
ystem in rat liver selects precursors
umably diglycerides) that contain
3. In contrast, the system that forms
om CDP choline preferentially forms
6-PC.

ce the PE is labeled with ^{14}C-22:6n3,
molecules can be converted to PC by
ntial methylation with *S*-adenosylme-
ine. It has been observed that ^{14}CH$_3$-
ionine is incorporated quickly and se-
ely into rat liver 22:6n3-PC *in vivo*
1). The combined actions of phospho-
s and transacylases (22) could also
the transfer of ^{14}C-22:6n3 from PE to

rat liver evidently selects 22:6n3-con-
g precursors for incorporation into PE
r than PC or the other lipid classes, and
bel later appears in PC. Docosahexae-
acid thus behaves distinctly differently
the other important fatty acids in rat
These observations suggest that the
on of 22:6n3 also is different from
of the other fatty acids.

nmary. ^{14}C-docosahexaenoic acid, pre-
by administration of ^{14}C$_1$-linolenic
o essential fatty acid-deficient rats, was
ed from liver phospholipids and con-
d to methyl docosahexaenoate, sp act 8
dpm/mg, purity about 90%. This was
rted to the free acid and injected into rats. ^{14}C-docosahexaenoic acid was incorporated mainly into carcass and liver. In liver, radioactivity appeared most rapidly in PE and later in PC. This behavior is unlike the incorporations of 16:0, 18:0, 18:1n9, 18:2n6, or 20:4n6, which are all incorporated most quickly into PC. Most of the radioactivity was recovered as 22:6n3, although considerable activity was present in C-16 and C-18 chains.

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Restricted Replication of Herpes Simplex Virus in Neural Cells (39384)

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(Introduced by C. W. Fishel)

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An intriguing property of the herpes simplex viruses (HSV) is their ability to establish a latent infection *in vivo*. Recent studies have demonstrated that the site of HSV persistence in animals and man is the neural elements of ganglia (1-4). During the course of latent infection the replication of the virus is restricted but an appropriate stimulus causes a shift in the host-virus relationship in the neural tissue, ultimately resulting in the replication of the virus and the recrudescence of a clinical infection (5, 6).

The efficiency of HSV replication *in vitro* varies with the host cell. For example, dog kidney cells and C6 cells fail to support (or actively restrict) the replication of HSV (7, 8). In addition, cells may respond to HSV infection with the production of interferon (7, 9, 10). Aurelian and Roizman (11) have documented the alternating suppression of the synthesis of interferon and HSV directed macromolecules during the cycle of infection in canine kidney cells. Thus, restrictive modulation of HSV replication may be the result of interferon production. This study sought to further characterize the interaction between HSV and C6 cells, which were derived from a glioma (12), specifically relating the cycle of infection to the potential cellular response of interferon synthesis.

Materials and methods. Cells and medium. The glioblastoma cell line, C6, purchased from American Type Culture Collection, was obtained from Dr. R. Grasso, University of South Florida. The rabbit skin (RS) and HeLa cell lines were obtained from Dr. L. McLaren, University of New Mexico. The composition of the medium was described in a previous report (13).

Viruses. Herpes simplex type 1 strain Seibert (HSV-1) was propagated in RS cells and concentrated by polyethylene glycol "precipitation" (14). The HSV was quantitated by plaque formation in RS cells employing an overlay which contained 0.5% methyl cellulose (15). Newcastle disease virus (NDV) was propagated in fertile hen's eggs and quantitated by plaque formation (13). Vesicular stomatitis virus (VSV) was propagated in and quantitated by plaque formation in C6 cells employing an overlay medium which contained 0.5% Noble agar.

Total HSV-1 content. Cell monolayers, infected at a multiplicity of infection (m.o.i.) of 10 plaque-forming units (PFU) per cell, were frozen (−85°) at the times indicated for the different experiments. The samples were then thawed, duplicates were pooled, and the cells were removed by centrifugation (800 *g*; 8 min). Cells were resuspended in 1 ml of the supernatant fluid and sonicated for 30 sec (20,000 cps). The remainder of the supernatant fluid was then added and the virus content was determined.

Assay for poliovirus in the presence of HSV-1. Monolayers which were infected with HSV-1 and superinfected with polio RNA were frozen (−85°) at the times indicated for each experiment, and subsequently thawed. Trition X-100 was added to the mixture of medium and cells to a final concentration of 0.5% which disrupted the cells, released the poliovirus, and inactivated the HSV-1. Poliovirus was quantitated by plaque assay on HeLa cells (16).

Interferon determinations. Medium alone or medium containing sonicated infected cells was acidified to pH 2, incubated at 4° overnight, and then neutralized (17). Dilutions of these samples were prepared in fresh medium and incubated with cells overnight. These monolayers were then challenged with approximately 100 PFU of VSV. The next day the monolayers were stained (18) and the plaques were counted. One unit of interferon was defined as the reciprocal of the sample dilution which resulted in a 50% decrement in plaque number relative to controls.

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ron preparation. Interferon was d by the NDV infection (m.o.i. = 6 cells. The medium was decanted r postinfection (hpi), acidified, and ently assayed for interferon activity ibed above.

RNA preparation. Infected HeLa re frozen, thawed, and then soni- rupted after infection by poliovirus. material was removed by centrifu- 800 *g*; 8 min) and the virus was by centrifugation (120,000 *g*; 90 he virus was resuspended in 0.02 *M* ite buffer, pH 7.1, and passed a 9.5 × 0.8-cm column of DEAE previously equilibrated with the ffer. Fractions containing poliovirus oled and sodium dodecyl sulfate was o a final concentration of 0.25%. sted protease (Sigma Chemical) was 100 μg/ml final concentration) and ple was incubated at 37° for 30 min. iple was then extracted twice with lumes of phenol, twice with equal of chloroform, and once with twice le volume of ether. The sample was ilyzed against 1 liter of phosphate l saline (PBS) for 24 hr at 4°.

RNA infection. Cells to be infected ished three times with Hank's bal- ilt solution (BSS; 19) and incubated iin with 1 ml of PBS containing 500 E dextran. The PBS was decanted, were exposed to the polio RNA for and then the original tissue culture was replaced. Determination of the ty of the polio RNA preparation ieved by plaque assay on HeLa cells the final medium added contained n agar and 30 m*M* $MgCl_2$. Incuba- polio RNA samples with ribonucle- μg; 20 min) reduced the infectivity reparation to less than 10 PFU/ml.

s. The temporal aspects of a single HSV-1 replication in C6 and RS re examined. A comparison of these on cycles revealed the inefficient ion of HSV-1 in C6 cells as shown in The productive cycle of infection in indicated a 4-hr eclipse phase fol- y the rapid synthesis of virus. By , the eclipse phase noted in C6 cells onged, through 12 hpi and was followed by an increase in HSV-1 levels. In addition, the adsorption of HSV-1 to both C6 and RS cells was equivalent as determined by the quantity of virus per 10^6 cells immediately following the adsorption period (Fig. 1, 0 hr). Thus, the low virus yield in C6 cells (1 PFU/cell) compared with 420 PFU/RS cell at 48 hpi reflected the restrictive nature of the HSV-C6 interaction.

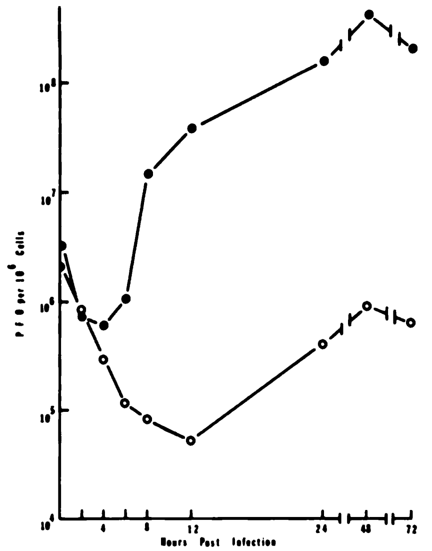

FIG. 1. Replication of HSV-1 in RS and C6 cells. Monolayers were infected with HSV-1 (m.o.i. = 10) as described above. At the times indicated, duplicate samples were frozen (−85°). All samples were subsequently processed as described in the Methods and the total virus content was determined (15). ●- - - -●, RS cells; ○- - - -○, C6 cells.

Since the production of interferon has been previously shown to suppress HSV directed biosynthetic metabolism (11), the relationship between interferon production and HSV-1 infection of C6 cells was examined. Medium from HSV-1 infected C6 cells as well as medium containing the total intracellular contents were assayed for interferon. In each instance, no interferon activity could be demonstrated in any of the samples of HSV-1 infected C6 cells tested (Table I). However, the infection of cells with NDV did result in the production of interferon. These data indicated C6 cells have the genetic capability to synthesize this antiviral protein, but they either failed to produce interferon in response to HSV-1 infection or synthesized quantities which

TABLE I. INTERFERON CONTENT IN MEDIUM AND INFECTED C6 CELLS.[a]

	Interferon titer (units/ml) Cells infected with:	
Hours postinfection	HSV-1	NDV
0, 2, 4, 6, 8, 12, 24, 48, 72	<5	
24		300

[a] Monolayers of C6 cells were infected with HSV-1 or NDV (m.o.i. = 10). At the times indicated the interferon content in the mixture of cells and medium was determined as described in the Methods.

could not be detected by the assay employed.

If low levels of interferon were responsible for restricting the replication of HSV-1 in C6 cells, then these cells might be expected to be refractory to superinfection by a second virus. This possibility was tested by monitoring the replication of infectious polio RNA which was used to superinfect C6 cells at different times post HSV-1 infection. Preliminary experiments indicated treatment of uninfected C6 cells with 5 units/ml of C6 interferon resulted in a 95% decrease in poliovirus production (data not shown). The results indicated that poliovirus synthesis was not appreciably inhibited through 10 hpi (Table II). However, by 24 hpi the decrement in poliovirus production in HSV-1 infected C6 cells was equivalent to that observed by 3.5 hpi in RS cells. While the levels of restriction of polio RNA replication were comparable, the time post HSV-1 infection when the restriction was manifested differed with the host cells.

The physiologically restrictive state of the C6 cells was further examined by determining the effect of exogenously added interferon on the replication of HSV-1. Monolayers received interferon before or immediately after HSV-1 adsorption and the total HSV-1 content was determined at 24 hpi. The results indicated 5 units/ml of C6 interferon had no effect on the yield of HSV-1 even when added 19 hr before the infection of the cells (Table III). Pretreating the C6 cells with 50 units/ml of interferon did result in a substantial decrement in HSV-1 yield. However, 50 units/ml of interferon failed to inhibit HSV-1 replication when it was added immediately after virus adsorption.

TABLE II. EFFECT OF HSV-1 INFECTION ON THE REPLICATION OF POLIO RNA IN C6 AND RS CELLS.[a]

Cell line	Time post HSV-1 adsorption of polio RNA superinfection (hr)	Poliovirus yield (% 0-hr yield)
C6	0	100[b]
	4	134
	10	67
	24	4
RS	0	100[c]
	3.5	9
	6.5	2

[a] Monolayers were exposed to HSV-1 (m.o.i. = 10) and virus adsorption proceeded for 2 hr at room temperature. At the times indicated, the monolayers were washed three times with BSS and superinfected with polio RNA as described in the Methods. At 24 hr post polio RNA infection, the cells were lysed with Triton X-100 and total poliovirus content was determined (16).

[b] 100% = 2.5 × 10^5 PFU/ml.

[c] 100% = 2.5 × 10^4 PFU/ml.

TABLE III. EFFECT OF EXOGENOUSLY ADDED INTERFERON ON THE YIELD OF HSV-1 IN C6 CELLS.[a]

Time of interferon addition (hpa)[b]	Interferon concentration (units/ml)	HSV-1 yield (% of control)
Control	None	100[c]
0	5	93
−19	5	98
Control	None	100[d]
0	50	135
−19	50	16

[a] Monolayers were divided into three groups. One group received interferon 19 hr before (−19) as well as immediately after HSV-1 adsorption. The second group received interferon only after HSV-1 adsorption (0), and the third group received no interferon (control). The cells were infected with HSV-1 (m.o.i. = 10) and at 24 hpi, the monolayers were processed as described in the Methods, and the total HSV-1 content was determined (15).

[b] hpa: hr postadsorption.

[c] 100% = 2.5 × 10^6 PFU/10^6 cells.

[d] 100% = 5.6 × 10^6 PFU/10^6 cells.

Discussion. The restrictive nature of the HSV-1 C6 cell interaction was evident from the diminished replicative capability of the virus in these cells. The maximum HSV-1 yield in C6 cells at 48 hpi was equal to or slightly below the quantity of virus which was present immediately following virus adsorption (0 hpi). These observations differ from the results in a previous study which reported a substantially lower quantity of HSV-1 in C6 cells at 48 hpi (8). The reason

ifference is not obvious and may be
ariation in the HSV-1 strains em-
n the two studies. However, the
ıce of HSV-1 adsorption to both
:S cells indicated that the restrictive
ım(s) regulating HSV-1 replication
ells occurred at the intracellular

fection of cells by HSV generally
result in the production of large
s of interferon (20). In addition,
ıction of interferon has been shown
tiplicity dependent, with maximum
›n occurring at a m.o.i. of 10 (11).
, no interferon activity was ob-
the composite of cells and media
V-1 infected C6 cells even though
have the genetic capability to pro-
:rferon, the m.o.i. employed was
a sensitive challenge virus (VSV)
in the interferon assay.

RNA was employed as a probe to
e whether HSV-1 infected C6 cells
ed a state of viral refractoriness in
ıce of producing readily detectable
s of interferon. This state might be
chieved since interferon synthesis
ortly following the transcription of
n messenger RNA (21, 22). Thus,
inhibition of polio virus yield when
ıfected C6 cells were superinfected
may be a consequence of the estab-
of an interferon-mediated viral re-
state. However, the data indicated
ɔle decrements in poliovirus yield
erved in C6 and RS cells superin-
24 and 3.5 hr post HSV-1 infec-
ɔectively. In addition, the RS cells
ı productive cycle of HSV-1 repli-
'ith no evidence of interferon pro-
data not shown). Thus, the inhibi-
oliovirus replication was probably
t of HSV directed interference be-
inhibition was manifest during the
e eclipse phases in C6 and RS cells,
ıs the time previously reported to
iated with HSV interference of
s replication (23). These data fur-
:ct upon the inefficient nature of
-1 C6 interaction as the HSV-1
ble by 10 hpi to establish control of
/nthetic processes in these cells
uld preclude the complete synthe-
iovirus.

The pretreatment of C6 cells with 50 units/ml of interferon demonstrated the susceptibility of the HSV-1 to the antiviral protein. The unrestricted replication of the HSV-1 in the presence of 50 units/ml of interferon added after virus adsorption reflected the relative resistance of this virus to interferon as well as the importance of the temporal relationship between the addition of exogenous interferon and the development of the refractory state to HSV-1. However, if interferon were produced in HSV-1 infected C6 cells, the level must be less than 5 units/ml. This amount of interferon did not inhibit the yield of HSV-1 in C6 cells even when the cells were pretreated with 5 units/ml of interferon 19 hr before HSV-1 infection. Thus, the data presented in this report suggest that interferon is not responsible for the restrictive replication of HSV-1 in C6 cells. The nature of the biochemical events associated with the restricted cycle of HSV-1 infection of C6 cells is currently under investigation.

Summary. The relationship between the replication of HSV-1 in C6 cells and an interferon-mediated state of viral refractoriness was studied. The cycles of HSV-1 replication in RS and C6 cells reflected the restrictive nature of the HSV-1 C6 interaction and indicated that the restrictive event(s) occurred intracellularly. However, interferon activity could not be demonstrated in the composite of medium mixed with the total intracellular contents from HSV-1 infected C6 cells from 0 through 72 hpi. The lack of a viral refractory state was also reflected in only a slight inhibition at 10 hpi of the replication of poliovirus RNA which was used to superinfect HSV-1 infected C6 cells. Furthermore, the exogenous addition of 5 units/ml of interferon supplied either before or after HSV-1 adsorption as well as 50 units/ml added after virus adsorption failed to affect the production HSV-1 in C6 cells. These observations suggest that interferon is not mediating the restrictive replication of HSV-1 in C6 cells.

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GS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 307-311 (1976)

ition of Intestinal Absorption of L-Phenylalanine *in Vivo* by L-Alanine (39385)

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active transport of L-amino acids by
ammalian small intestine and the in-
ions between individual compounds
een studied by several groups of in-
tors (1–4). Mutual inhibition in the
nal transport of aromatic amino acids,
as L-tyrosine, L-tryptophan, and L-
lalanine (L-Phe), in *in vitro* systems
when there is a relatively large ratio
inhibitor to the inhibited amino acid
. This line of investigation has been
cted also in other laboratories, partic-
those interested in the elucidation of
utual interference between L-Phe and
ine (L-Ala) (4), and galactose, L-Phe,
Ala (3).

of potential clinical interest to search
ernative approaches to the established
treatment of phenylketonuria, con-
of a very limited intake of L-Phe,
at reducing plasma L-Phe levels. In
esent studies we have explored the
of action and kinetics of L-Ala inhibi-
n L-Phe intestinal transport in an *in*
stem using isolated, open segments of
per jejunum in anesthetized rats. L-
as shown to impair a nonspecific
acid carrier system and substantially
L-Phe absorption rates, as well as
of other amino acids, when the L-
nino acid ratio was at least 10:1. The
on L-Phe transport appeared compat-
ith a noncompetitive type of inhibi-

erials and methods. Female Wistar
eighing between 200 and 250 g, were
n the experiments. They received a
rd pelleted feed (purina Lab Chow,
n Purina Co.) and water *ad libitum*.
was withdrawn the afternoon prior to
orning of the perfusions in order to
accumulation of semidigested resi-
the upper intestinal tract. The rats
nesthetized with 1.2 g/kg body weight
hane (Aldrich Chemical Co.) administered intraperitoneally. The abdominal cavity was opened by a midline incision and the small intestine cannulated below the ligament of Treitz. A 20-cm long segment was utilized, avoiding carefully any damage to the mesenteric circulation. The intestinal contents were washed out with two 10-ml portions of warm saline. The proximal junction was attached to a peristaltic pump (Harvard Apparatus, Model 1201), and perfused at a rate of 0.20–0.22 ml/min. The distal end was allowed to drain by gravity into graduated tubes. The solutions perfused were Krebs–Ringer bicarbonate buffers containing variable amounts of the amino acids or other substances tested (Sigma Chemical Co.). Polyethylene glycol, mol wt 3000–3700 (PEG, "Carbowax 4,000," Allied Chemical Co.) was used as a nonabsorbable marker at a concentration of 600 mg/100 ml. Tracer amounts of appropriate ^{14}C- or ^{3}H-labeled amino acids (New England Nuclear Corp.) were added such that the perfusion fluids contained 20,000 to 50,000 dpm/ml, in addition to the unlabeled metabolites. The identity of the radioactive substance was tested in the buffers and in perfusates by thin-layer chromatography of 0.025-ml samples on silica gel plates with butanol:acetic acid:water (60:15:25) as a solvent (7). The solutions were kept isotonic by adjusting the concentration of sodium chloride. The buffers were bubbled with a O_2:CO_2 (95:5) gas mixture (Matheson Gas Products) and maintained at 37° during the experiments. Each rat was perfused with two different buffers containing or omitting L-Ala in order to minimize biological variability. The sequence of the buffers perfused was randomized. Fractions were collected every 15 min for 1 hr, after a 45-min equilibration period. A 30-min wash interval was allowed before collecting samples for a second hour. Actual pumping rates were measured before and after the

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eserved.

completion of the experiment. The intestinal segment was removed immediately after sacrificing the animal by exsanguination and measured on a flat surface to the nearest mm.

All fractions collected were centrifuged at 600 *g* for 10 min to separate debris and cells, and 0.50-ml aliquots of the supernatants were transferred to scintillation vials, mixed with 10 ml of a water-compatible scintillator, and counted with efficiency better than 50% for ^{14}C or 20% for ^{3}H. PEG was assayed by a turbidimetric method (8) and the ratio of concentrations indicated the water flux to and from the intestine. The concentration of the solute was determined by isotope dilution. No detectable chromatographic alteration of the radioactive label was observed after passage through the intestine, determined to take only 9 to 10 min. The rates of absorption for each fraction were calculated according to the following formula (9):

$$\left(S_{bf} - \frac{PEG_{bf}}{PEG_{s}} \times S_{s}\right) \times \frac{1000 \times \text{Flow rate (ml/min)}}{\text{Intestinal segment length (cm)}} = \frac{\text{nmole}}{\text{min} \times \text{cm}},$$

where S_{bf} and S_{s} were the m*M* concentrations of the amino acids in the perfusing buffer and in the sample, and PEG_{bf} and PEG_{s} were the concentrations of PEG in the buffer and the sample, respectively. Additional extensive details of the perfusion technique and the analytical methods have been published previously (10, 11). The values reported here are means ± SE obtained from perfusions of 4 to 12 rats. Student's *t* test was applied to determine the statistical significance of the difference of means (12).

Results and discussion. The absorption rates of L-Phe, when perfused either at 1 or 2 m*M* concentrations, were affected by the simultaneous presence of L-Ala only when the ratio of L-Ala to L-Phe was 10 to 1 or higher (Fig. 1). These concentrations of L-Phe tested were selected because the carrier system for the intestinal transport of the amino acid is not saturated (9, 10) and because they were low enough to allow the addition of relatively large amounts of possible inhibitors without reducing excessively the sodium concentration. The presence of a 20 to 1 molar ratio of L-Ala to L-Phe decreased L-Phe absorption rates by 27.8% for a 1 m*M* perfusate and by 43.7% in the 2 m*M* L-Phe solution.

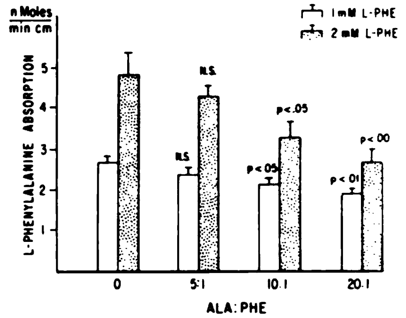
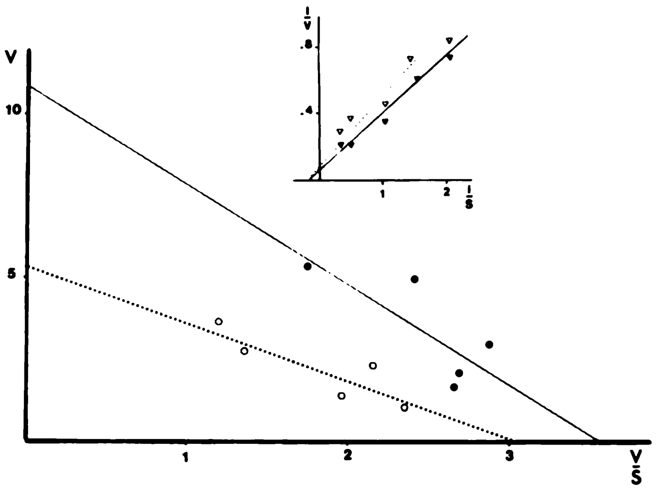


FIG. 1. Inhibition of L-Phe intestinal absorption by L-Ala. The experiments were performed with 1 m*M* (open bars) or 2 m*M* L-Phe (dotted bars) in the presence of no inhibitor, and of L-Ala in various molar ratios, as described in the Materials and Methods section. Each bar represents the mean ± SE of 16 to 48 values, corresponding to 4 to 12 rats perfused. The significance of the differences as compared to the data of the same concentrations without inhibitor was calculated by a nonpaired Student's *t* test (12).

A *V* versus *V*/*S* plot was drawn from data obtained from L-Phe absorption rates in 0.5 to 3.0 m*M* concentrations in the absence and in the presence of 40 m*M* L-Ala (Fig. 2). It has been shown that the *V* versus *V*/*S* representation is superior to the *1*/*V* versus *1*/*S* Lineweaver–Burk plot, which was included in the illustration for comparative purposes (13–15). These graphs show the rates of absorption *(V)* for different concentrations of the substrate L-Phe *(S)* with or without the simultaneous presence of L-Ala. In order to increase the accuracy of the kinetic analysis, the arithmetic mean between the initial and final concentrations of the perfusates was used for a numerical calculation of V_m and K_t to minimize the error which may arise from consumption of the substrate (16). The K_t derived from the slope of the *V* versus *V*/*S* plot in the absence of L-Ala was determined to be 3.0 m*M*. The apparent K_t value, in the presence of 40 m*M* L-Ala, was not significantly different, indicating a noncompetitive type of inhibition.



2. V versus V/S plot using the mean intestinal transport rates (V) and the concentration of the substrate 'S) in the perfusates. In the insert, the same data were used for the plotting of a Lineweaver–Burk plot. circles or triangles represent points obtained for intestinal transport rates in the absence of L-Ala. Open ls are values derived from data in the presence of 40 mM L-Ala. The regression lines were drawn by the juares method (12).

same conclusion could be obtained the Lineweaver–Burk plot. A statisti- umerical estimate of the K_t applying inson's fine adjustment calculations gave a value of 2.40 mM (SE ± 1.35) uffers without L-Ala, and 1.87 mM (SE 12, t = 0.39, N.S.) in the presence of L- under the conditions stated above. , using the same analytical procedure, $'_m$ for the perfusates containing no L- ;ave a value of 10.08 nmole/min × cm ± 2.74), while the V_m for the buffer ining 40 mM L-Ala yielded a rate of (SE ± 0.68, t = 2.40, $P < 0.05$). e figures for the intestinal transport af- constants K_t are somewhat lower than derived from *in vivo* studies in humans ut are similar to those reported for *in* experiments with rabbit intestinal mu- which were 2.7 mM (2) and 3.5 mM espectively. The type of inhibition ex- by L-Ala on L-Phe intestinal transport was ascertained according to Dixon's ic procedure (17) for absorption rates Phe at 1 and 2 mM concentrations in resence of increasing levels of L-Ala. A idental K_i value of 48 mM was ob- d.

r own results agree with previous stud- ı substrate interrelationships in the ac- ransport of neutral amino acids across the intestinal mucosa (18–21). The L-Phe/L-Ala pair has been extensively explored. Findings indicating that the maximal influx of L-Ala was greater than that of L-Phe suggested to previous investigators that these two amino acids could not be sharing all possible mechanisms of intestinal transport and that L-Ala and L-Phe influxes were mediated by two separate carrier systems which did not differ in their abilities to bind these substances (2, 4, 18). Another important consequence of these studies was the clear discrimination between binding and membrane translocation as two independent processes. These concepts are now confirmed with an *in vivo* experimental model. The similarities between the K_t obtained for L-Phe in the rat *in vivo* system and the *in vitro* rabbit intestinal mucosa, if not an indicator of identity in the binding and transport capacities for that amino acid under different physiological conditions, underscore the comparable order of magnitude for transport constants determined in dissimilar experimental settings.

The effects of a constant 20 to 1 molar ratio of L-Ala to other essential and structurally related amino acids on their intestinal absorption rates are listed in Table I. The L-Ala excess produced significant declines in the rates of intestinal transport for (L-) ly-

TABLE I. INTESTINAL ABSORPTION RATES OF L-AMINO ACIDS IN THE PRESENCE OF A 20:1 MOLAR RATIO FOR L-ALANINE:L-Amino Acid.

Substance	Absorption rates (nmole/min × cm) Without L-Ala	 With L-Ala
Lysine[a]	11.76 ± 0.64[b]	6.92 ± 0.36[c]
Histidine	9.16 ± 0.28	4.79 ± 0.45[c]
Methionine	10.23 ± 0.34	8.41 ± 0.60[d]
Arginine	8.10 ± 0.65	9.08 ± 0.35
Leucine	9.38 ± 0.50	9.32 ± 0.94
Isoleucine	8.10 ± 0.65	8.02 ± 0.25
Valine	6.36 ± 0.36	5.71 ± 0.41
Tryptophan	4.10 ± 0.30	3.34 ± 0.42
Tyrosine	5.12 ± 0.48	4.56 ± 0.32
Threonine	7.27 ± 0.57	6.48 ± 0.43

[a] All (L-) amino acids were perfused at a 2 m*M* concentration. L-Alanine (L-Ala) was present at a 40 m*M* concentration.

[b] The absorption rates represent means ± SE; N = 16 or more determinations for each substance tested, in the absence or in the presence of the inhibitor. The significance of the differences was checked by a Student's *t* test (12).

[c] $P < 0.001$.

[d] $P < 0.05$.

sine, histidine, and methionine. Conversely, 40 m*M* L-Ala had no effect on the absorption of (L-) arginine, leucine, isoleucine, isoleucine, valine, tryptophan, threonine, and tyrosine, when perfused at 2 m*M* levels. These results may indicate that the common transport mechanism for neutral amino acids is also operative for positively charged amino acids and that the proportion of the translocation load for these substances is based toward the common carrier. The heterogeneity in the transport capacity of dibasic amino acids, suggested by the variety of cystinurias and dibasic aminoacidurias described in the literature (22), encourages a similar speculation for neutral amino acids, that is, the coexistence of common and specific carrier systems, either as separate entities or as discrete active sites on the same proteins.

In another series of experiments, the effects of galactose were tested by itself or in conjunction with 40 m*M* L-Ala (Table II). While this amino acid consistently produced a decline in the absorption rates of L-Phe, galactose alone had only minimal consequences. However, the simultaneous perfusion of both substances produced an additive effect and a reduction of the absorption rates to almost half the baseline values. This action could not be attributed to a lower sodium concentration since an equivalent molarity of mannitol had no action on L-Phe absorption rates. In addition, the absolute requirement of the amino group in the alpha position was confirmed, since β-alanine in comparable concentrations had no influence on the transport of L-Phe by the small intestine of the rat.

Since there is general agreement to assume the existence of specific sites or transport systems in addition to common carriers for neutral amino acid translocation (1–6), simultaneous inhibition of both the specific and the nonspecific translocation pathways would be required for a virtual shut off of L-Phe transport. In the experiments reported here, specialized mechanisms appeared responsible for the bulk of intestinal amino acid absorption, since a large molar excess of a potential inhibitor such as L-Ala produced only limited effects.

It is difficult to discriminate under experimental conditions *in vivo* the role of separate carrier systems available for the intestinal transport of L-amino acids. Kinetic studies reveal the physiological options existing at the intestinal mucosa surface. The lack of a competitive type of inhibition for L-Ala over L-Phe can be taken as evidence of the multiplicity of active sites available for the translocation of neutral amino acids. A more effective control of L-Phe absorption may come from the use of selective, specific, antagonists such as β-thienyl-DL-alanine or L-dihydroxyphenylalanine, as we have shown in previous studies (23), in addition

TABLE II. EFFECTS OF VARIOUS SUBSTANCES ON THE INTESTINAL ABSORPTION RATES OF 2 m*M* L-PHE.

Additions	Absorption rates (nmole/ min × cm)	Change (%)
None	6.22 ± 0.30[a]	–
40 m*M* L-Ala	4.23 ± 0.43	−32.0[b]
40 m*M* galactose	5.52 ± 0.56	−11.3
40 m*M* L-Ala + 40 m*M* galactose	3.19 ± 0.36	−48.8[c]
80 m*M* mannitol	6.96 ± 0.10	+11.9
40 m*M* β-alanine	6.16 ± 0.32	−1.0

[a] Conditions, number of determinations, and significance, as in Table 1.

[b] $P < 0.01$.

[c] $P < 0.001$.

oncompetitive agents of low toxicity
as L-Ala. Therefore, unless a consider-
reduction of both specific and nonspe-
intestinal transport systems can be
ved by the use of appropriate antago-
the clinical significance of the effects
r described will remain very limited in
:.

nmary. The intestinal transport of L-
rom 1 and 2 m*M* solutions in the pres-
of variable concentrations of L-Ala was
ed with an *in vivo* technique utilizing
n long jejunal segments in anesthetized
The simultaneous perfusion of L-Ala
ited the absorption of L-Phe when the
r ratio L-Ala:L-Phe was 10:1 or higher.
esults suggested a noncompetitive type
ibition under the experimental condi-
described. Other essential amino acids
as L-lysine, L-histidine, and L-methio-
at 2 m*M* concentrations were also sig-
ntly inhibited by 40 m*M* L-Ala. These
are consistent with the presence of two
: transport mechanisms for neutral
o acids in the intestinal mucosa: a com-
one which can be blocked by large
ntrations of L-Ala, and a specific site
rier which is not affected by this inhib-

thank the technical assistance of Susan A.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 312-317 (1976)

Effect of Norepinephrine on the Ovine Umbilical Circulation (39386)

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The autonomic nervous system has a role in the regulation of the placental blood flows in the sheep. One of the most distinctive responses of the uterine vascular bed is a vasoconstriction when exposed to norepinephrine (3). Barrett *et al.* (2) showed that the fetal renal and umbilical circulations both reacted to methoxamine with a decreased flow in the face of an increased arterial pressure, thereby indicating that alpha receptors are present in these vascular beds. In contrast to this report Novy *et al.* (9) observed that the umbilical circulation of the fetal sheep does not show a vasoconstriction in response to norepinephrine. This result is difficult to interpret as it is unlikely that norepinephrine would fail to elicit responses from alpha receptors if these receptors are present in the umbilical circulation. In an attempt to clarify this question we have performed a series of experiments in which we examined the effect of norepinephrine on the renal and placental circulations of near-term sheep fetuses.

Methods. Nine pregnant sheep with gestational ages ranging from 120 to 138 days were used in this study (term = ca. 145 days). The sheep were sedated with Nembutal (5 mg/kg) intravenously and given spinal anesthesia (4–8 mg tetracaine hydrochloride).

Polyvinyl catheters (i.d. 0.58 mm) were placed into a superficial artery of the maternal hind limb and advanced 20 cm at which point the tip was in the maternal femoral artery. The abdomen was opened and catheters of the same type were placed in a fetal hind limb artery and vein in the manner that is described by Rankin *et al.* (10). A catheter was attached to the fetal hind limb so that amniotic fluid pressures could be measured. The maternal left ventricle was catheterized with a combination PE200 catheter encompassing a polyvinyl catheter in a manner previously described (12). An electromagnetic flow transducer was placed on one of the middle uterine arteries in a manner which is described by Raye *et al.* (13). The flow transducer leads, maternal femoral catheter, and fetal catheters were brought to the flank through a subcutaneous tunnel and sealed in a pouch. The maternal carotid catheter was sealed in a bandage around the neck. Each ewe received penicillin 300,000 units and streptomycin 0.5 mg im each day following surgery.

Protocol. One to three days postoperatively the ewes were placed in a stanchion in the laboratory and permitted to stabilize for 2 hr. The fetal venous and arterial and maternal arterial blood pressures were monitored by blood pressure transducers (Statham P23Db) with the zero reference at the level of the scapulo humeral joint. The flow in the middle uterine artery was recorded via a Micron RC1000 flowmeter and all signals were recorded on a Beckman R411 recorder. Fetal and maternal bloods were drawn on several occasions for blood gas determinations. All experiments were performed on animals in which the fetal arterial pH was greater than 7.3.

At the end of the stabilization period the distribution of the maternal and fetal blood flows was measured with 25-μm radioactive microspheres (3M Company) using the method of Makowski *et al.* (8). Spheres labeled with separate isotopes were simultaneously injected into the fetal hind limb vein and the maternal left ventricular catheters. The catheters (dead space = 0.2 ml) were then flushed with 3 ml of saline. At the same time arterial blood was drawn from the fetal hind limb artery and the maternal femoral artery into 2 m of PE205 tubing. The withdrawal rate was 2.1 ml/min for fetal blood and 3.8 ml/min for maternal blood. The withdrawal continued for 45 sec after the completion of the microsphere injection. The blood contained in the PE205 tubing

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erved.

n forcibly ejected into wide glass g vials for subsequent assay.

pinephrine (Levophed, Winthrop) in of dosages was then injected into the nb vein catheter and the catheter with 2 ml of saline. One and a half later the distribution of the fetal ternal blood flows was measured vith spheres labeled with a further topes. Precautions taken during the g of catecholamines were based on ommendations of Barton *et al.* (3). reparation was given microspheres r different radioactive labels, these ^{1}Ce, ^{85}Sr, ^{125}I, and ^{95}Nb.

s possible to give several injections pinephrine to each sheep in order to the pressor and flowmeter re- although only two simultaneous ob- ns of the maternal and fetal blood uld be made with the microspheres. e cases the normal response of the vasculature was established by ob- the effect of a test injection of 50 μg inephrine given via the left ventric- heter on the uterine blood flow as d with the flowmeter. The alpha re- of the fetal circulation were then with phenoxybenzamine (Dibenzy- administering 25 mg of this agent period of 10 min through the hind in catheter. After a delay of 45 min ction of norepinephrine was then the fetus, and the fetal and mater- onses were recorded. The uterine ure was again tested with 50 μg of ephrine administered via the left lar catheter to determine whether this circulation was affected by the benzamine.

e end of the experiment the animal rificed, and the maternal kidneys, nd uterus were removed for radioac- y. The uterus was opened and the ns, membranes, and fetal kidneys d as previously described (11). The were homogenized and aliquots of ogenate were placed in wide, glass vials for assay. The integrated arte- d samples and vials containing the amples were counted on a three- Nuclear Chicago 1185 gamma and data reduction performed via an interactive terminal in our laboratory. Details of this technique have been described previously (6). The resistance of the maternal organs was calculated by dividing the maternal arterial pressure by the blood flow to that organ. The resistance of fetal organs was calculated by subtracting the fetal venous pressure from the fetal arterial pressure and dividing the result by the organ blood flow. This procedure does not differentiate between changes in the hepatic resistance and changes in the resistance of the placental vascular beds but Barrett *et al.* (2) have shown that methoxamine does not cause a constriction of the hepatic vasculature. We have therefore assumed that changes in the pressure in the cord veins will be closely mirrored by changes in inferior caval pressure. The statistical significance of the differences between control and treatment means were evaluated by the paired Student's *t* test.

Results. Fetal responses. The responses of the fetal circulations of seven sheep are presented in Table I. It can be seen that the dosage of norepinephrine varied widely. We introduced this variation in an attempt to construct a dose response curve relating the amount of norepinephrine injected to the fetus and the maternal response, but were not able to observe a consistent relationship between the amount of norepinephrine and the response of the mother. The fetal arterial pressure always increased secondary to the injection of norepinephrine to the fetus. Mean fetal arterial pressure in the control period was 36 mm Hg which rose to 67 mm Hg, a change which was highly significant ($P < 0.001$). The fetal venous pressure rose secondary to the injection of norepinephrine into the fetus. The control value was 5.5 mm Hg and the test value was 11 mm Hg ($P < 0.001$). We could observe no change in the amniotic fluid pressure when norepinephrine was given to the fetus. The transplacental pressure drop was calculated as the fetal arterial minus fetal venous pressure. The mean control value for this parameter was 30.4 mm Hg and it rose to 56 mm Hg secondary to the injection of norepinephrine to the fetus. This difference is significant ($P < 0.001$).

The umbilical blood flow changed when

TABLE I. THE FETAL WEIGHT, AMOUNT OF NOREPINEPHRINE INJECTED, FETAL ARTERIAL MINUS VENOUS PRESSURES AND VASCULAR RESISTANCES OF THE FETAL COTYLEDONARY, RENAL, AND PLACENTAL MEMBRANE CIRCULATIONS AS MEASURED WITH RADIOACTIVE MICROSPHERES BEFORE (CONTROL) AND AFTER (TEST) EIGHT INJECTIONS OF NOREPINEPHRINE TO SEVEN FETUSES.

Sheep and injection number	Fetal weight (kg)	Dose injected (μg/kg)	Pressures (mm Hg) Fetal arterial − Fetal venous		Vascular resistances (mm Hg × min/ml × kg)					
					Umbilical		Renal		Membrane	
			Control	Test	Control	Test	Control	Test	Control	Test
1	3.6	27	37	60	0.220	0.583	6.38	9.00	6.07	4.80
3a	2.5	80	27	56	0.165	0.255	3.75	14.93	3.55	14.00
3b[a]	2.5	120	27	55	0.135	0.374	4.22	13.03	2.81	13.75
4	4.0	50	29	50	0.181	0.385	3.41	14.66	1.35	3.62
5	3.5	29	37	56	0.214	0.675	4.81	11.64	2.19	19.31
6	2.6	77	27	55	0.172	1.309	2.60	21.15	1.71	28.95
7	2.3	22	28	54	0.215	1.200	2.92	18.49	2.15	24.55
8	3.9	26	32	60	0.123	0.870	2.22	27.03	2.12	33.33
Mean	3.1	54	30	56	0.178	0.706	3.79	16.24	2.74	17.79
SD ±	0.7		4.3	3.2	0.037	0.390	1.35	5.78	1.50	10.79
			$P < 0.001$		$P < 0.003$		$P < 0.001$		$P < 0.004$	

[a] Two sets of observations were made with sheep 3.

norepinephrine was injected into the fetus. The control value for the umbilical blood flow was 177 ml/min/kg, and the value obtained after the injection of norepinephrine into the fetus was 105 ml/min/kg fetus. This change was significant ($P < 0.001$). The fall in umbilical blood flow was rendered more impressive when it was considered in terms of the rise in arterial minus venous pressure. The umbilical vascular resistances are given in Table I and changed from a control mean of 0.178 mm Hg × min/ml × kg fetus to 0.706 mm Hg × min/ml × kg fetus after norepinephrine ($P < 0.003$). It can be concluded that the administration of norepinephrine to the fetus causes a vasoconstriction in the umbilical circulation. The renal flow changed from a mean value of 8.75 ml/min/kg to 4.43 ml/min/kg fetus ($P < 0.004$). The decrease in flow and the increase in blood pressure indicated vasoconstriction. There was also vasoconstriction of the fetal membranes in which the flow changed from a mean value of 13.2 ml/min/kg to 5.4 ml/min/kg ($P < 0.008$). Data pertaining to the resistance of the fetal renal and placental membrane vascular beds are given in Table I.

An example of the changes in fetal arterial and venous pressure that we observed is given in Fig. 1. The output of the electromagnetic flowmeter and the maternal arterial pressure are also displayed in the figure. At the right of the figure the unmeaned signals are given at a higher paper speed.

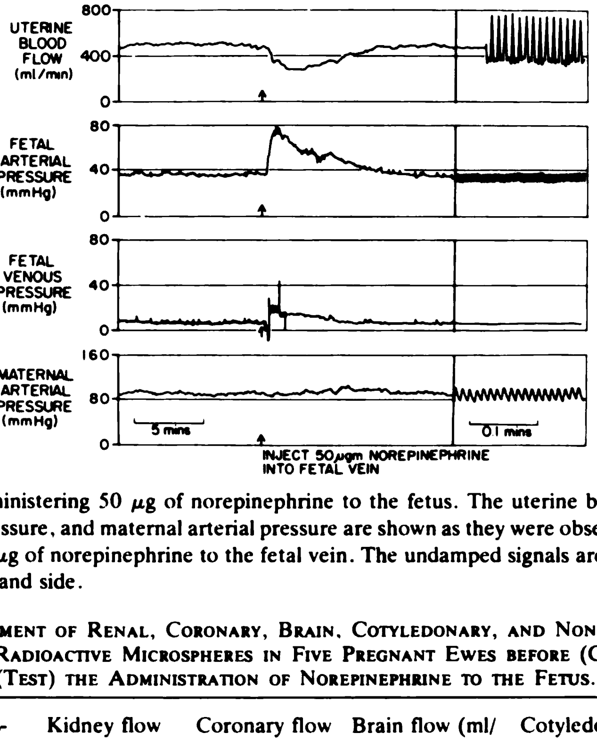
In the five animals to which phenoxybenzamine was given to the fetus, we could observe no fetal pressor response nor change in fetal venous pressure secondary to the injection of norepinephrine. The fetal arterial pressure either remained constant or decreased slightly.

Maternal responses. Thirty-five injections of norepinephrine were given to the fetuses of nine sheep. In 29 cases the uterine blood flow was observed to fall subsequent to the injection of norepinephrine to the fetus. In the six cases in which no responses were observed, five were injections given subsequent to phenoxybenzamine blockade and one was an injection given to a fetus which was severely acidotic. An example of the normal response is shown in Fig. 1. It can be seen that the uterine blood flow begins to fall with the onset of the fetal pressor response and remains at lower than normal levels for the duration of the fetal pressor response. In five cases the regional maternal blood flows were quantitated with radioactive microspheres before and during this response. The changes in the cotyledonary and noncotyledonary uterine flows were compared with the responses of the maternal kidney, heart, and brain. These results

Table II. It can be seen that nephrine was given to the fetus significant change in the mater- ressure, maternal kidney flow, onary flow, nor maternal brain ncotyledonary uterine flow did significantly. The only signifi- as that the cotyledonary blood from a mean value of 778 ml/ e of 611 ml/min at the peak of . This change is significant (P =

t of the electromagnetic flow- the pressure recordings were late the uterine vascular resistance before and after the postblockade injection of norepinephrine in the fetuses of sheep 2, 3, 4, 5, and 7. The ratios of uterine resistance before and the uterine resistance after giving norepinephrine to these fetuses were 1.00, 1.17, 0.94, 1.00, and 0.83, respectively, with a mean value of 1.0. These data indicate that there was no change in the uterine vascular resistance subsequent to the injection of norepinephrine to the fetus after the fetal circulation was blocked with phenoxybenzamine.

The response of the maternal placental blood flow to norepinephrine administered to the mother was observed in the same five



ct of administering 50 μg of norepinephrine to the fetus. The uterine blood flow, fetal arterial enous pressure, and maternal arterial pressure are shown as they were observed in sheep number 7 on of 50 μg of norepinephrine to the fetal vein. The undamped signals are shown in an expanded e right-hand side.

Measurement of Renal, Coronary, Brain, Cotyledonary, and Noncotyledonary Blood E with Radioactive Microspheres in Five Pregnant Ewes before (Control) and after (Test) the Administration of Norepinephrine to the Fetus.

terial pres- (mm Hg)		Kidney flow (ml/min)		Coronary flow (ml/min)		Brain flow (ml/min)		Cotyledonary flow (ml/min)		Noncotyledonary uterine flow (ml/min)	
n-l	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test
8	88	454	465	318	117	62	53	786	695	148	139
1	81	394	580	47	58	27	39	658	355	119	183
4	72	930	1010	154	167	51	60	908	886	251	260
0	100	540	527	225	232	62	57	522	399	137	178
8	72	798	781	–	–	–	–	1017	721	324	425
4	83	623	673	186	143	51	52	778	611	196	237
0	12	250	223	114	74	17	9.3	196	227	88	114
NS		NS		NS		NS		$P < 0.023$		NS	

sheep. When 50 μg of norepinephrine was given to the maternal left ventricle of these animals before fetal blockade, the uterine blood flow decreased an average of 67%. After the fetal circulation was blocked with phenoxybenzamine, the administration of norepinephrine to the mother caused the uterine flow to decrease an average of 64%. The difference between these two responses was not significant.

Discussion. Archie *et al.* (1) have discussed in depth the validity of the radioactive microsphere technique in the measurement of organ blood flows. Buckberg *et al.* (5) have described the possible errors in the technique and point out that at least 400 microspheres should be in each sample if statistical errors are to be acceptably low. We had at least 400 microspheres in each of our integrated arterial samples and the use of five aliquots of the organ homogenates meant that we had at least 400 microspheres in the total tissue mass assayed from each organ.

It is probable that the lowest dose of norepinephrine used was maximal or supramaximal because all fetuses responded with strong vasoconstriction in the umbilical and renal vascular beds. The dose dependent nature of the response would therefore not be apparent in the range used in this study. The phenoxybenzamine blockade of the fetal circulation was apparently effective at 45 min after the administration of this drug, because norepinephrine did not elicit a pressor response at that time even though complete alpha receptor blockade in the adult requires up to 2 hr.

The fetal pressor response to norepinephrine is in accordance with the results obtained by Novy *et al.* (9). The changes in peripheral resistance that we observed in the fetal circulation are not in agreement with the established literature. We know of no previous description of the effect of norepinephrine on the fetal kidney but we observed a vasoconstriction in this organ when norepinephrine was given to the fetus. This observation confirms the presence of alpha receptors in the fetal kidney as postulated by Barrett *et al.* (2). We found that the umbilical vascular bed responded to norepinephrine with vasoconstriction which is also *in agreement* with the work of Barrett *et al.* (2) but not in agreement with the work of Novy *et al.* (9).

There are three possible reasons for this disagreement. In the first case the dose levels were different. Novy *et al.* used lower dose levels than were used in our studies and the responses may not be observable in the lower range. In the second case, Novy *et al.* worked with acute preparations which may have already been under maximum catecholamine stimulation. A third possible reason for the discrepancy is that prostaglandins seem to act as modulators of autonomic effector sites. Prostaglandins of the E series tend to diminish the cardiovascular responses to norepinephrine (4, 7). In a previous series we found that in adult sheep the norepinephrine-induced uterine placental vasoconstriction was severely depressed for over an hour after the administration of prostaglandin E_2. This depression of alpha adrenergic activity persisted long after the blood pressure and heart rate had returned to normal. Novy *et al.* gave PGE_2 and norepinephrine to each animal and the responses to norepinephrine would have been abnormally small in those cases where PGE_2 administration preceded the norepinephrine administration.

The changes in the uterine blood flow that we observed secondary to the injection of norepinephrine to the fetus are purely local phenomena. Concomitant changes are not seen in the maternal blood pressure or in the maternal brain, kidney, coronary, or even noncotyledonary uterine flow.

The mechanism of this change in uterine blood flow is not as obvious as would first appear. The first conclusion upon examining the data is to ascribe the change in the uterine flow to the transplacental flux of small amounts of norepinephrine which locally affected alpha recep tors in the maternal uterine circulation. If this had happened then giving phenoxybenzamine to the fetus would have either had no effect on the uterine response to fetal norepinephrine or blocked part of the uterine vasculature. We observed that phenoxybenzamine abolished the uterine response to fetal norepinephrine but caused no change in the uterine response to maternal norepinephrine. We conclude that the response of the uterine vascular bed to the injection of norepineph-

ı the fetus may not be due to the direct
of norepinephrine on the uterine vas-
re.

ı*mary*. The administration of norepi-
ne (50 μg/kg) to the fetuses of nine
caused an increase in fetal arterial
re (Control = 30 mm Hg; Test = 56
g; $P < 0.001$) and vasoconstriction in
:al kidney (Control = 3.79 mm Hg ×
1 × kg; Test = 16.24 mm Hg × min/
‹g; $P < 0.001$), placental membranes
:ol = 2.74 mm Hg × min/ml × kg;
17.79 mm Hg × min/ml × kg; $P <$
), and fetal cotyledons (Control =
mm Hg × min/ml × kg; Test = 0.706
g × min/ml × kg; $P < 0.003$). These
indicate the presence of alpha recep-
these organs. The uterine blood flow
bsequent to the injection of norepi-
ne to the fetus and this response was
ıed by phenoxybenzamine blockade
: circulation of four fetuses even
ı we could demonstrate no phenoxy-
nine blockade in the uterine circula-
' these animals.

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Comparison of the Effects of *N*-Ethylmaleimide and Iodoacetamide on Red Blood Cell Membranes[1] (39387)

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The importance of free sulfhydryl groups in maintaining red blood cell (RBC) membrane structure and function has been extensively studied, employing *N*-ethylmaleimide (NEM) and other agents (1–3). NEM produces a rapid reduction in RBC filterability (4) and a loss in RBC viability (5). RBC membrane polypeptides reacting with [^{14}C]NEM have been compared with those reacting with [^{14}C]iodoacetamide (IA) by polyacrylamide gel electrophoresis containing sodium dodecyl sulfate (PAGE SDS) (6, 7). IA at low concentrations produces specific labeling of glyceraldehyde-3-phosphate dehydrogenase (G3PD), whereas NEM at similar levels or IA at higher concentrations binds with less specificity to many membrane components (7). Here we compare the effects of NEM with those of IA on RBC membrane polypeptides, G3PD, ATP levels, and filterability.

Methods. Using aseptic technique, heparinized whole blood was freshly obtained from normal healthy donors, centrifuged (1200 *g*, 10 min), and the plasma, buffy coat, and top one-fifth of the RBC removed. Similar efforts to remove white cells were made after three subsequent washes of the RBC with Dulbecco's phosphate buffered saline (PBS) (8). Cell counts were performed on 50% RBC suspensions with a Coulter model S cell counter and the RBC were observed under phase microscopy. Aliquots of RBC were measured in this standard freshly washed state, so that volume changes induced by incubation would not be a factor in measurements reported. Washed RBC were incubated with two volumes of 10 m*M* IA or 10 m*M* NEM in PBS, for 2 hr at 37° to obtain maximal effect with less than 1% hemolysis (5). For labeling experiments [^{14}C]NEM and IA 10 mCi/mmole were used (New England Nuclear). RBC filtration employed the method of Teitel *et al.* (4) employing 9-cm Schleicher and Schuell No. 589 white band filter paper at 25° using twice-washed cells, at 50% hematocrit in PBS. RBC membranes were prepared by the method of Dodge *et al.* (9) as modified by Fairbanks *et al.* (10). To measure total membrane protein, RBC were hemolyzed with exactly 60 volumes of 0.005 *M* phosphate buffer, pH 8, centrifuged for 30 min at 80,000 *g*, and the pellet collected and brought to standard volume. Total protein was measured by the Lowry procedure (11), hemoglobin by the benzidine method (12), and nonhemoglobin protein reported as the difference. Prior to electrophoresis the protein was dissolved in 1% SDS, containing 1% mercaptoethanol, and heated to 100° for 2 min to destroy proteases. SDS PAGE of from 10–300 μg of protein was performed on 0.5 × 10-cm gels. The gels, containing 5% acrylamide in 0.1% SDS and 0.1 *M* phosphate buffer, pH 7, were run at 25° (10 mA/tube), stained for proteins with Coomassie blue (10), and the relative amounts of protein components were determined on a Canalco gel scanner. Autoradiography of [^{14}C]NEM- and [^{14}C]IA-labeled polypeptides was performed on longitudinally sliced gels, which were dried on filter paper (13), and wrapped with Kodak no-screen film for 28 days. The autoradiograms were scanned and compared with the PAGE SDS protein scan. G3PD was assayed on isolated membranes, one unit of actively reducing 1 μmole/min of nicotinamide–adenine dinucleotide at 25° (14), and ATP concentrations were determined enzymatically (15) using a Gilford spectrophotometer.

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TABLE I. Effects of RBC Incubation in PBS, NEM, and IA.[a]

	G3PD[b] (units/mg membrane protein)	ATP[b] (μmole/dl RBC)	Nonhemoglobin protein[c] (g × 10^{-13}/cell)	1/2 Filtration time[b] (min)
PBS (0 time)	1.8 ± 0.3	96.25 ± 3.5	9.9 ± 0.3	4.5 ± 1
PBS (2 hr control)	1.4 ± 0.1	94.0 ± 1.0	10.0 ± 0.8	4.3 ± 1
N-Ethylmaleimide	0	56.8 ± 2.2	13.1 ± 0.7	∞
Iodoacetamide	0	55.8 ± 3.5	9.2 ± 0.4	5.0 ± 1

[a] Two volumes of 10 m*M* inhibitor, 37°, pH 7.4, 2 h).
[b] Mean from four individual determinations ± SD.
[c] Mean from six individual determination ± SD.

Results. Table I compares the G3PD ac- ity, ATP levels, and RBC membrane pro- in content with RBC filtration of control BC, RBC incubated for 2 hr at 37° in PBS, in two volumes of 10 m*M* NEM or IA. oth NEM and IA completely inactivated e G3PD, and produced a similar 40% duction in ATP levels. While this decrease ATP was identical, NEM markedly in- bited RBC filtration whereas IA did not able I). Thus, the decrease in filtration te with NEM cannot be ascribed to effects ATP. The modification by NEM of the BC membrane was confirmed by the in- eased amounts of nonhemoglobin protein r RBC membrane isolated ($P < 0.005$, the Student's *t* test). Membranes from EM-treated RBC were noted to pack bet- r than those from control cells, perhaps lated to the cell shrinkage observed with EM (5), and thus are contained in a aller volume in the one-step hemolysis ocedure, and require fewer centrifuga- ons to yield white ghosts.

The sequential changes in membrane lypeptides induced by NEM treatment e shown in Fig. 1. The polypeptide bands e numbered as in Fairbanks *et al.* (10). e lowest curve represents the PAGE SDS membranes from unincubated control BC, and the remaining curves the effects incubation with 20 μmole NEM/ml RBC r 15, 30, and 120 min at 37°. Marked anges are clearly present at 15-30 min d included the apparent increase in bands ready detected in control membranes, ch as band 6 previously identified as 3PD (16) and band 4.2, the appearance of w bands such as those between polypep- les 2 and 3, and a complex increase in nds in the region of 4.5.

The relation of the protein changes induced by [^{14}C]NEM at 2 hr to binding of this inhibitor is shown in Fig. 2. It is apparent that many of the NEM-produced polypeptides are heavily labeled with [^{14}C]NEM.

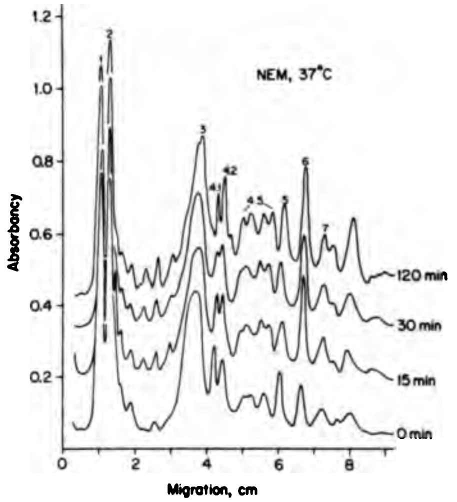

FIG. 1. Effect of NEM (20 μmole/ml RBC, 37°) for 0, 15, 30, and 120 min on PAGE SDS. 120 μg of protein was applied in each case. Note the progressive appearance of many new polypeptide bands produced by NEM. The 15-, 30-, and 120-min scans have been displaced upwards on the ordinate to aid visualization.

The NEM-induced accumulation of new bands occurred only in intact cells. Treatment of isolated membranes with NEM either with or without prior exposure to 1% mercaptoethanol (100°, 2 min) yielded no additional bands, as shown in Fig. 3. These findings are consistent with the cytoplasmic origin of the additional nonhemoglobin protein recovered in the membrane fraction after NEM treatment of intact RBC. Since deliberately increasing the amount of white blood cell contamination from 400/μl to 40,000/μl failed to increase the rate or extent of NEM-induced effects, these changes

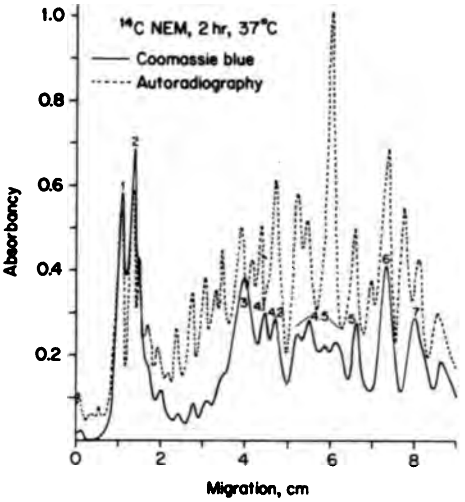

FIG. 2. [^{14}C]NEM-labeled membranes: comparison of polypeptide stain and autoradiograph. RBC were labeled with [^{14}C]NEM (20 μmole/ml RBC, 2 hr, 37°), and 300 μg of solubilized membrane protein was applied to PAGE SDS gels.

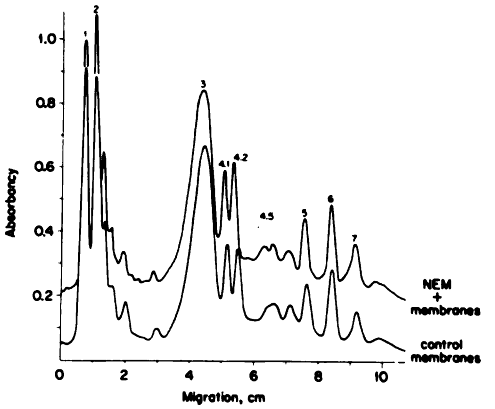

FIG. 3. Lack of effect on isolated membranes of NEM (20 μmole/ml packed membranes, 60 min, 37°) (upper scan), compared to control membranes, incubated in buffer alone, 60 min, 37° (lower scan).

almost certainly do not arise from leukocyte proteinase action. Moreover, no release of a proteinase by NEM was demonstrable using the detector Azocoll (Calbiochem); however, activation of membrane-fixed proteinases could not be ruled out (17, 18). Omitting calcium from the PBS had no effect on the NEM-induced changes, unlike sterile incubation of glucose-deprived RBC *in vitro* (19).

Figure 4 compares the protein scan of the stained [^{14}C]IA-labeled polypeptides on PAGE SDS with the scan of the autoradiograph. The relative concentration of polypeptides on the scan is similar to that of fresh RBC seen in the lowest curve in Fig. 1, except an increase in band 6 and a possible increase in band 4.2. The autoradiograph shows that these bands are also particularly heavily labeled with [^{14}C]IA and that the labeling of other bands is generally proportional to their protein content, as reported by others with these concentrations of [^{14}C]IA (7). When RBC were treated with IA for 1 hr, the changes shown in Fig. 4 appeared. When the IA-treated cells were then incubated with NEM for an additional hour, the membranes isolated, and electrophoresed, the multiple new membrane bands in NEM-treated RBC membranes became apparent.

Discussion. The correlation of the filtration rate with changes in the protein content and composition of the RBC membranes provides some evidence that changes in the isolated membranes may apply to the intact cell. Thus, a decrease in filterability of RBC occurs with NEM which alters the PAGE SDS and protein content of the membrane significantly, and not with IA which does not. With NEM the rate of the decrease in

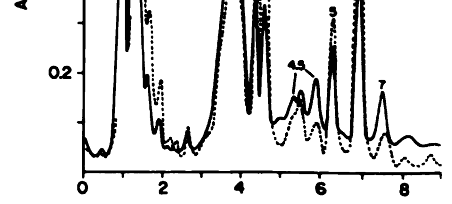

FIG. 4. [^{14}C]IA-labeled membranes: comparison of polypeptide stain and autoradiograph. RBC were labeled with [^{14}C]IA (20 μmole/ml RBC, 2 hr, 37°) and 300 g of solubilized membrane protein applied to PAGE SDS.

filterability (4) and appearance in new bands (Fig. 1) is similar. Teitel *et al.* (4) observed that the amount of NEM required to alter filterability had to exceed, mole for mole, RBC glutathione content (2 μmole/ml). Similarly, more than 2 μmole NEM/ml RBC were needed before there were any changes in the polypeptides on PAGE SDS. The lack of significant modification of the PAGE SDS polypeptide scan makes IA labeling of reactive sulfhydryl groups more readily interpretable than [^{14}C]NEM labeling. Since pretreatment with IA did not block subsequent NEM-induced changes, it is presumed that NEM combined at additional protein sites.

The mechanisms causing the polypeptide alterations in the red cell membrane have been only partially clarified. The increase in band 6 (G3PD) caused by its specific combination with IA or NEM may be explained by stabilization of the membrane-bound state of the enzyme by the inhibitor, with prevention of substrate-induced dissociation (20). How generally this type of mechanism might apply to other membrane components (such as band 4.2) is not known. The correspondence of the [^{14}C]NEM reactivity of a polypeptide and its appearance in the membrane fraction suggests a direct chemical relation between these events. For example, NEM may convert cytoplasmic components to a denser or more hydrophobic configuration by its direct addition to a side group or by disruption of tertiary protein structure. Such changes might result in the decreased solubility of the proteins and their subsequent recovery in the membrane fraction. Additional effects, such as increased susceptibility to an endogenous protease following such configurational changes, have not been entirely ruled out.

Summary. Although both NEM and IA completely inhibited G3PD activity and decreased RBC ATP levels 40%, IA produced little effect on RBC filterability whereas NEM greatly reduced the filtration rate. IA caused only an isolated increase of band 6 (G3PD) and no change in membrane protein content. However, the decreased filterability of NEM-treated RBC was associated with significantly increased membrane-associated nonhemoglobin protein which appeared as a complex array of polypeptide bands on PAGE SDS.

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Immunoregulatory Consequences of Vitamin Deficiencies on Background Plaque-Forming Cells in Rats (39388)

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The detrimental effects of specific vitamin-deficiency states on the immune response have been known for some time (1). The pronounced impairment of the immune system has been well documented in both pyridoxine- and pantothenic acid-deficient rats (1). Defects in immune capacity in such deficient animals can be attributed neither to general inanition nor to impaired reticuloendothelial function in antigen distribution, but rather to decreased numbers of hemolytic antibody-producing cells as measured in plaque-forming cell assays (2) in rats responding to sheep erythrocytes (3, 4).

In an effort to extend these studies to animals injected with other antigens, and in order to approach questions regarding mechanisms of regulation of immune responses in general, and mechanisms of the immunological defects seen in vitamin-deficient rats in particular, studies were undertaken using targets in addition to sheep erythrocytes to evaluate the immune capacity of rats with specific vitamin deficiencies. In this report, we present results of the effects of riboflavin, pyridoxine, and pantothenic acid deficiencies upon background plaque-forming cells in nonimmunized rats.

Materials and Methods. Male, weanling albino rats of the Holtzman strain (Holtzman Co., Madison, Wisc.) were housed individually in wide-meshed, screen-bottom, suspended cages. They were weighed weekly and experiments were initiated 6 to 14 weeks after arrival at the laboratory.

Production of specific vitamin deficiences. All animals were fed a basal, semipurified diet *ad libitum* (4). In addition, each animal received a daily vitamin pill (4). Each of the pills fed to the control animals supplied adequate supplements of all of the B vitamins known to be required by the rat. For the riboflavin-, pyridoxine-, and pantothenic acid-deficient groups, only the respective vitamin was omitted from the control pill.

Assays for hemolytic plaque-forming cells. The following types of erythrocytes were used as targets in the direct hemolytic plaque-forming cell (PFC) assay (2): sheep red cells (SRC) from individual sheep were obtained in Alsever's solution from either Earl Cole, Green County, Pa.; Flow Laboratories, Rockville, Md., or Sacks' Farm, Evans City, Pa.; human O+ erythrocytes were obtained in ACD solution from the Central Blood Bank, Pittsburgh, Pa.; pooled rat red cells in Alsever's solution were obtained by cardiac puncture of donor normal rats of the same Holtzman strain as used in experiments.

Rats were sacrificed by a blow on the head and bled by cardiac puncture. Spleens and, sometimes thymuses, were removed aseptically, weighed, and dissociated by being pressed through a stainless steel wire mesh (2). Single cell suspensions were prepared (2); and aliquots of the resulting single cell suspensions containing 10^6 to 10^8 cells, depending on targets to be used, were subjected to hemolytic plaque assays (2) in 15 × 100-mm petri dishes. Preliminary experiments were run using more than one concentration of cells in order to determine appropriate cell concentrations. Base layers were not used in order to increase assay sensitivity; plaques were counted under 30× magnification using a dissecting microscope and indirect fluorescent light. A minimum of triplicate plates were counted for each type of red cell target used. Only those

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plaques fulfilling criteria for true PFC (2) were counted.

Results. The results of 10 experiments[3] involving rats with specific deficiencies of pantothenic acid, pyridoxine, or riboflavin are summarized in Fig. 1 and Tables I and II. The background PFC in nonimmunized rats directed against the selected erythrocyte targets were higher in the vitamin-deficient rats compared with controls in each of three specific vitamin deficiencies studied (Fig. 1). In contrast, we observed a markedly depressed response of PFC to immunization with SRC in riboflavin- and pantothenic acid-deficient rats.[4] The latter findings are in accord with previous observations in pyridoxine- (3) and pantothenic acid-deficiencies (4).

The increase in the numbers of background PFC per 10^8 spleen cells in the deficient groups was apparent despite the significant differences in whole body weight as well as in spleen weights in the deficient rats compared with control rats (Table I). Furthermore, not only is there an increase in background PFC per 10^8 spleen cells in the deficient groups, but there is an increase in the total numbers of such cells per spleen, as shown in Table II. This is true even through the spleens of deficient rats are strikingly smaller than control spleens (Table I). The ratio of spleen weight to body weight does not vary significantly between deficient groups and their respective controls. The number of cells per milligram of spleen also remains constant (1 million cells per mg) for each group. Thus, there is a definite overall increase in background PFC and not merely a relative increase of these cell populations compared with other spleen cells.

In experiments in which PFC in thymuses were also determined on the same erythrocyte targets as used in spleen PFC assays, the numbers of background PFC in thymuses of vitamin-deficient rats were also equal to or higher than the numbers of PFC in comparable control rats.

The increase in background PFC was found in PFC directed against all targets tested. The reactivity of PFC to SRC, human O+, and rat red cells (R) probably cannot be attributed to a shared antigen reactivity. The SRC used in these studies came from three different suppliers, and although SRC blood groups were not determined, only one blood type among seven blood groups in sheep appear to be related to H of man (5). Moreover, in experiments in which vitamin-deficient rats as well as control rats

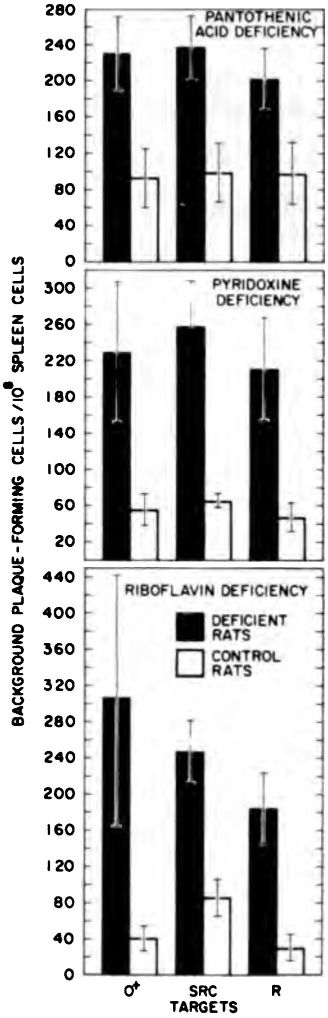

FIG. 1. Background plaque-forming cells (PFC) per 10^8 cells in spleens of six groups of rats against three different erythrocyte targets, human O+ red cells (O+) sheep red cells (SRC), rat cells (R). The numbers of rats in each group are those shown in Table I. PFC are shown as arithmetic means. Vertical bars represent ±1 SE.

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[3] In each of 10 experiments, cell suspensions from experimental and control rats were run in parallel under identical laboratory conditions.

[4] Koros, A. M. C., Axelrod, A. E., Hamill, E. C., and South, D. J., unpublished data.

TABLE I. BODY WEIGHTS AND SPLEEN WEIGHTS OF VITAMIN DEFICIENT AND CONTROL RATS.

Group	Number of rats per group	Rat weight (grams)[a]	Spleen weight (milligrams)[a]
Pantothenic acid-deficient	31	130.0 ± 9.0	425.9 ± 24.4
Controls	30	262.8 ± 12.9	836.3 ± 41.1
Riboflavin-deficient	17	73.0 ± 3.7	225.4 ± 12.0
Controls	12	214.8 ± 20.7	786.0 ± 58.6
Pyridoxine-deficient	21	102.1 ± 8.0	357.9 ± 17.7
Controls	25	237.3 ± 8.9	868.7 ± 49.6

[a] Arithmetic mean ± 1 SE.

TABLE II. PLAQUE-FORMING CELLS PER TOTAL SPLEEN IN VITAMIN-DEFICIENT AND CONTROL RATS.[a]

	PFC per total cells per spleen[b]		
	Erythrocyte targets		
Group	O+	SRC	R
Pantothenic acid-deficient	908.8 ± 199.2	858.5 ± 149.3	818.2 ± 168.5
Controls	489.6 ± 220.3	516.8 ± 200.3	488.2 ± 185.2
Riboflavin-deficient	614.4 ± 219.6	608.9 ± 138.3	340.1 ± 52.5
Controls	277.1 ± 124.9	562.6 ± 132.9	172.8 ± 64.2
Pyridoxine-deficient	788.7 ± 412.5	757.4 ± 219.7	577.6 ± 154.2
Controls	301.5 ± 84.5	408.2 ± 66.9	245.0 ± 57.3

[a] The number of rats per group is the same as shown in Table I.

[b] Values based on actual cell counts (10) expressed as arithmetic mean ± 1 SE.

were inoculated with a single large dose of SRC, the control immunized rats responded dramatically to SRC when spleens were assayed on SRC targets, but exhibited no increase over background PFC when assayed on human O+ or R erythrocytes.[4] The numbers of postimmunization anti-SRC PFC in these immunized control animals were comparable to those found earlier (3, 4).

Discussion. Although the significance of an increased number of background PFC in certain vitamin-deficient states is not known presently, it is tempting to speculate that background PFC may reflect a unique regulatory parameter in both normal and immunologically impaired animals for the following reasons: (i) Suppressive 7S specific anti-SRC antibody, which markedly inhibits the appearance of postimmunization PFC directed against SRC, has no effect on background PFC directed against SRC (6). (ii) Background PFC directed toward different haptens appear at predictable times in a predictable sequence in ontogeny for particular strains of inbred mice (7). (iii) Background PFC exist in germ-free mice in numbers comparable to those found in conventional mice (8) and are probably not merely an indicator of prior exposure to cross-reacting bacterial antigens. (iv) The morphology of background PFC also differs from postimmunization PFC. Although the majority of direct (19 S) PFC directed against SRC are either blast cells, large lymphocytes, or plasma cells, whose numbers vary depending on the time after antigen injection, the background PFC are small lymphocytes (9–11). (v) Only a small percentage (<5%) of background PFC have been shown to be involved in DNA synthesis, compared with 55% of Day 4 postimmunization PFC which are actively synthesizing DNA (10). The background PFC thus appear to have different rates of proliferation than postimmunization PFC.

The present study cannot attempt to define the function of background PFC in absolute terms, but does present a comparative survey of their existence in certain vitamin-deficient states in outbred rats. Categorization of background PFC as members of either B or T cell populations as described for mouse models (12) would require the use of inbred rats.

Increases in background PFC, especially autoimmune PFC, have been reported by others in NZB mice precedeing the onset of autoimmune disease (13, 14). Increases in

ound PFC have also been reported in
nice (15), and further elucidation of
le of background PFC might offer a
the postulated link between autoim-
diseases and aging (15).

ght of recent findings of the functional
geneity of lymphoid cell populations,
ally T cells (16), it is possible that in
ecific vitamin-deficient rat popula-
tudied in this report, there is an in-
in background PFC directed against
ecificities tested, whereas there is a
se in the precursor population(s) of
munization PFC directed against the
pecificities. Such a situation could be
the loss of a population of cells which
unction normally to suppress back-
l PFC as well as to cooperate with
sors of postimmunization PFC. Al-
the possibility cannot be excluded
e background PFC are direct precur-
f postimmunization PFC, it is sug-
from the current study as well as
(3, 4) that there may be a lack of a
population necessary for the increase
ber of PFC following antigenic stimu-
in vitamin-deficient animals even
the background PFC exist in com-
or even greater numbers in vitamin-
nt rats than in controls. Whether this
from an actual increase in the rate
iferation of background PFC in vita-
ficient animals or a selected survival
age of such cells only in vitamin-
nt animals is not known. Indeed
chanisms whereby the vitamin defi-
s in this study impair the immune
se are not known. Pyridoxine defi-
is the only one of the three studied
report that has been shown to affect
synthesis (17).

also possible that background PFC
e directed against different, cross-
g specificities rather than the same
rythrocyte specificities to which post-
ization PFC are directed. Neverthe-
e fact that all background PFC are
lly elevated in the three categories of
-deficient animals studied compared
ntrol animals suggests that the num-
background PFC are a parameter of
latory defect in all of the deficient
.

The finding of PFC against rat red cells may also be the result of a loss of a population of suppressive cells which are postulated to normally function to keep self-reactive cells in check (18, 19).

It is hoped that further studies into the nature of background PFC and their relation to postimmunization PFC may lead to a greater understanding of normal regulatory mechanisms involved in the immune response.

Summary. Background hemolytic plaque-forming cells (PFC) directed against three erythrocyte targets were measured in rats with three different, specific vitamin deficiencies: riboflavin, pyridoxine, and pantothenic acid, as well as in control rats. The numbers of background PFC were found to be elevated in all three vitamin-deficiency states compared with controls, whereas earlier studies showed that postimmunization PFC were markedly depressed in those deficiencies tested. The significance of these findings is discussed in terms of a possible loss of suppressor cells.

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THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 327-329 (1976)

n of Liver Plasma Membranes Using Lactoperoxidase: Effects on Adenylate Cyclase Activity (39389)

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(Introduced by J. I. Kitay)

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nt years, studies of receptors for
peptide hormones have focused on
cyclase activity and hormone
ites in isolated cells and purified
membrane preparations. These
ve provided major insights regard-
ysiology of hormone action. How-
tively little has been learned of the
properties of receptors or of the
mechanisms involved in hormone
nteractions. The major obstacle to
a more basic understanding of re-
their membrane location. While
rmation has been obtained with
d hormone-sensitive adenylate cy-
arations (1-3), receptors must ul-
e studied in their natural membra-
ronment.

toperoxidase-catalyzed iodination
d tyrosines has been used to label
mponents of plasma membranes
types of cells (4-6). The surface
of this enzyme suggested that it
vide useful information regarding
l arrangement of receptor compo-
in an intact membrane. This re-
nstrates the effects of iodination
purified plasma membranes from
n adenylate cyclase activity in the
s.

ls and Methods. Lactoperoxidase,
estimated purity 60%, was ob-
rom Calbiochem. Carrier-free
m Amersham/Searle. Acrylamide,
hylenebisacrylamide, and *N,N,*
methylethylenediamine were ob-
n Eastman.

membranes were prepared by a
on (7) of the procedure of Neville
ylate cyclase was assayed as de-
previously (7) except that
was separated from [^{32}P]ATP by
um oxide method devised by Ramachandran (9). Protein was measured by the procedure of Lowry *et al.* (10).

For amino acid analysis, liver membranes, 2 mg of protein, were hydrolyzed for 24 h at 100° in 6 *N* HCl. Amino acids were then estimated using a Spinco 120C amino-acid analyzer.

Results. Iodination of liver plasma membranes with lactoperoxidase has not been reported previously. As shown in Fig. 1 this procedure results in incorporation of iodine into several membrane proteins ranging from mol wt 20,000 to 100,000. The resolution of peaks of radioactivity is not adequate to determine the exact number or to compare extent of iodination with the quantity of protein in a given band.

The effects of iodination on membrane adenylate cyclase activity are illustrated in Fig. 2. The maximal glucagon and fluoride stimulated activities are reduced by 63 and 46%, respectively. The extent of incorporation of iodine is also shown in Fig. 2. Although full saturation was not obtained in this experiment, the incorporation of iodine clearly approaches a maximum under these conditions. Maximal effects on adenylate cyclase activity are seen with less than 10% of the total incorporation of iodine which could be obtained indicating that the affected component(s) of adenylate cyclase is particularly susceptible to iodination. The inhibition of adenylate cyclase is partial and no further inhibition is seen despite increased incorporation of iodine at higher KI concentrations. The effect on glucagon-stimulated activities is seen with less incorporation of iodine than is the effect on fluoride-stimulated activity.

Table I shows the effects of omitting each of the components of the iodination mixture and demonstrates that inhibition of adenylate cyclase activity is enzymatic and depend-

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d.

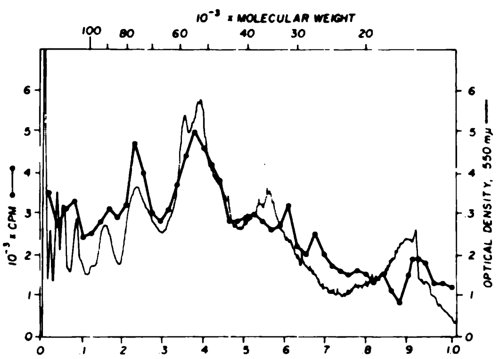

FIG. 1. Incorporation of ^{125}I into plasma membranes and resolution of labeled proteins by polyacrylamide gel electrophoresis. Plasma membranes, 1 mg of membrane protein, were suspended in 1 ml of 0.1 *M* Tris-HCl, pH 7.6, 10^{-6} *M* KI, 1 μCi $Na^{125}I$, and 10^{-6} *M* lactoperoxidase. The reaction was performed at ambient temperature and was initiated by addition of 5 μl H_2O_2 to give a final concentration of 0.3 μ/ml. Additional 5-μl aliquots of dilute H_2O_2 were added every 15 sec for a total of 2 min and the reaction mixture was placed on ice. Membranes were dissolved, dialyzed, and subjected to electrophoresis in sodium dodecyl sulfate polyacrylamide gels as described by Neville (11) using a pH-10 lower buffer. The gels were stained with Coomassie blue by the method of Fairbanks *et al.* (12). The stained gels were scanned at 530 nm in a spectrophotometer fitted with a linear transport device (Gilford Instruments). Parallel gels containing proteins of known molecular weight were used to calibrate the gels. The gels were fractionated with a Maizel gel extruder (Savant). The fractions were counted in a gamma scintillation counter.

ent on the presence of H_2O_2. The data show further that basal adenylate cyclase activity is inhibited by iodination and that the effect on glucagon-stimulated activity is on the maximal activity (10^{-6} *M* glucagon; (7)) rather than on the apparent affinity for glucagon estimated from the activity in the presence of 4×10^{-9} *M* glucagon.

Amino acid analysis revealed that the membrane used in this study contained 18 nmole tyrosine and 15 nmole histidine per mg of membrane protein. In addition to tyrosine, lactoperoxidase catalyzes the iodination of monoidotyrosine and histidine as free amino acids (13). However, it has not been established whether these amino acids are substrates in membranes. Consequently the number of iodine acceptors could be as low as 18 nmole or as high as 51 nmole per mg of protein. Since the maximum iodination achieved in the experiment described in Fig. 2 is 3 natoms per mg protein, this would correspond to 6 to 17% of the total potential iodine acceptors. The number required to produce maximal effects on adenylate cyclase activity is 10-fold less.

Discussion. The lactoperoxidase catalyzed iodination of membrane proteins has generally been used as a labeling procedure to study the disposition of proteins within a membrane. In this study lactoperoxidase is used as a probe of a specific membrane function, the glucagon receptor-adenylate cyclase system. The inhibition of adenylate cyclase produced by iodination indicates that a component of the receptor-adenylate cyclase system containing a tyrosine, or possibly a histidine, is susceptible to attack by lactoperoxidase.

A current hypothesis regarding the molecular organization of the glucagon receptor system is that it is composed of a catalytic subunit, adenylate cyclase, on or near the inner surface of the plasma membrane and a separate regulatory subunit on or near

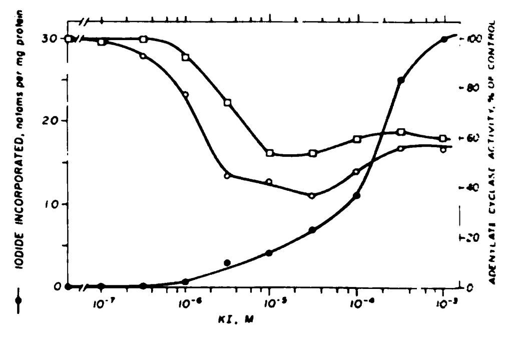

FIG. 2. Effects of iodination on membrane adenylate cyclase activity. Membranes were iodinated as described in Fig. 1 in the presence of varying concentrations of KI and in the presence and absence of a tracer quantity of $Na^{125}I$. Incorporation of iodine into the membranes was estimated by layering 0.1-ml aliquots of the reaction mixtures containing ^{125}I over 0.3 ml of 1% bovine serum albumin in a microfuge tube. The membranes were sedimented for 5 min in the microfuge (Beckman). The supernatant was aspirated and the tube was rinsed once with water. The tip containing the membrane pellet was cut off and counted in a gamma counter. Adenylate cyclase activity in the membranes treated without ^{125}I was measured as described under Methods. Glucagon (—○—) was 10^{-6} *M*. Fluoride (—□—) was 15 mm. Values are expressed as percentage of no I^- control and are the means of triplicate determinations.

TABLE I. ADENYLATE CYCLASE ACTIVITY.[a]

Lacto-peroxidase	H_2O_2	KI	Basal	Glucagon (4×10^{-9} *M*)	Glucagon (10^{-6} *M*)	NaF 15 m*M*
			(nmoles per 10 min per mg protein)			
−	−	0	0.89 ± 0.02	2.69 ± 0.26	6.77 ± 0.12	2.02 ± 0.07
+	−	0	0.93 ± 0.01	2.57 ± 0.24	7.14 ± 0.14	2.27 ± 0.01
+	−	10^{-6}*M*	0.84 ± 0.12	2.90 ± 0.54	6.96 ± 0.09	2.10 ± 0.03
+	−	10^{-5}*M*	0.88 ± 0.01	2.35 ± 0.22	7.22 ± 0.08	2.11 ± 0.08
+	−	10^{-4}*M*	0.86 ± 0.09	2.17 ± 0.32	6.71 ± 0.03	2.13 ± 0.07
+	+	0	0.90 ± 0.01	1.88 ± 0.04	6.52 ± 0.10	2.05 ± 0.06
+	+	10^{-6}*M*	0.55 ± 0.04	1.21 ± 0.14	4.06 ± 0.15	1.42 ± 0.01
+	+	10^{-5}*M*	0.21 ± 0.05	0.36 ± 0.04	0.80 ± 0.05	0.48 ± 0.01
+	+	10^{-4}*M*	0.32 ± 0.01	0.32 ± 0.01	1.78 ± 0.04	0.75 ± 0.01
−	+	10^{-5}*M*	0.82 ± 0.02	2.61 ± 0.54	6.51 ± 0.13	1.82 ± 0.02

[a] Liver plasma membranes were incubated in the presence of absence of 10^{-6} *M* lactoperoxidase and varying concentrations of KI for 2 min at room temperature as described in Fig. 1. Water was added in place of H_2O_2 to the minus H_2O_2 controls. At the end of the iodination, the reaction mixtures were placed on ice and adenylate cyclase activity was measured in the presence of no addition, 4×10^{-9} *M* glucagon, 10^{-6} glucagon, or 15 m*M* NaF was measured as described under Methods. Values are the mean ± SE of triplicate determinations.

the outer surface to which glucagon binds. The inhibition of basal and fluroride-stimulated activities by iodination indicates that the catalytic subunit is affected. The incompleteness of the inhibition of adenylate cyclase by iodination may be due to the heterogeneity of membrane preparation. The liver membrane preparation is composed of a mixture of open sheets and closed vesicles (7). Adenylate cyclase on the inner surface of the vesicles may be protected from attack by lactoperoxidase.

The amino acid analysis indicates that no more than about 20% of the total iodinatable substrates (tyrosine, monoiodotyrosine, histidine) in the membrane are iodinated under the conditions employed here. The remainder are probably buried within the membrane and less accessible for iodination. The observation that maximal effects on adenylate cyclase occur with only about 10% of the total possible incorporation of iodine suggests that the affected component is very superficially situated in the membrane and is readily accessible for attack by lactoperoxidase. Neer has shown that solubilized renal adenylate cyclase has very little associated lipid or detergent and therefore is probably located on the surface of the membrane (3). The data presented here support this hypothesis. If this interpretation is correct, then the regulatory component(s) of the system must traverse the hydrophobic core of the membrane. The importance of membrane phospholipids in hormone sensitivity of adenylate cyclase has been amply demonstrated (1, 14).

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Quantitation of Human T-Lymphocytes in Blood: Use of Automatic Smearing Instrument for Preparing Fixed Slides of E-Rosettes[1,2] (39390)

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The presence of functionally distinct populations of lymphocytes (B and T lymphocytes) in the peripheral blood is generally recognized (1). Human T-lymphocytes are commonly identified as the lymphocytes that form rosettes with SRBC (E-rosettes) when incubated under appropriate physical conditions. Quantitation of E-rosettes is commonly performed in cell suspension using a hemocytometer (2). The disadvantages of this method are lack of permanent preparation, inability to precisely identify the morphology of the rosette-forming cells, and the necessity of counting the rosettes within a short period of time due to their fragile nature. To obviate the above limitations, smears of E-rosettes were prepared by an automatic smearer which yielded very satisfactory preparations with no significant destruction of the rosettes.

Materials and Methods. Ten normal volunteers, three patients with chronic lymphocytic leukemia, and one patient with Sézary syndrome were studied (Table I). Venous blood was obtained in heparinized plastic syringes. Lymphocytes, isolated by Ficoll-Isopaque method (3), were washed twice with phosphate-buffered saline (PBS) with 0.1% glucose and suspended at a concentration of 40×10^6/ml. Sheep red cells (SRBC) suspended in Alsever's solution (Grand Island Biological, New York) were washed twice in PBS and resuspended in PBS at a concentration of 3000×10^6/ml. Fetal calf serum (FCS) (Grand Island Biological, New York) was absorbed twice with SRBC at 37° for 30 min (800×10^6 SRBC/ml FCS) and heat-inactivated at 56° for 1 hr.

For hemocytometer assay, 0.5 ml of the lymphocyte suspension readjusted with PBS to 15×10^6/ml was mixed with 0.5 ml of washed SRBC diluted to 300×10^6/ml and 0.01 ml of absorbed and inactivated FCS in a round bottom 75 × 15-mm plastic tube. Following gentle agitation the mixture was centrifuged at 200 *g* for 10 min at room temperature prior to incubation at 4° for 16 to 20 hr. The incubation tube was gently rotated by hand to resuspend the cells. A Neubauer chamber was gently filled with the cell suspension using a Pasteur pipet and allowed to stand at 10° for 10 min. Five-hundred lymphocytes were counted under phase contrast at a magnification of 400.

For automatic smearing method 0.05 ml of lymphocyte suspension (2×10^6 cells) was mixed with 2.5 ml of medium 199 with 25 m*M* HEPES buffer (Grand Island Biological, New York) in a round-bottom 75 × 15-mm plastic tube. Following centrifugation at room temperature for 10 min at 500 *g*, the supernatant was drained off. To the cell pellet, 0.01 ml of the SRBC suspension (30×10^6 cells) and 0.01 ml of the absorbed, inactivated FCS were added. Following gentle agitation the mixture was incubated at 37° for 15 min, at 4° for 16 to 20 hr, and finally at room temperature for 1 to 2 hr. The incubation mixture was gently rotated by hand until no clumps were visible. About 0.01 ml of the incubation mixture was placed on a glass slide and smeared with the automatic smearer, Hemaprep (Geometric Data Corp., Wayne, Pa.), adjusted to the thickest smear setting. This instrument makes smear by pulling the slide

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[2] Written informed consent, as required by our Institutional Review Committee, was obtained from normal volunteers and patients.

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TABLE I. PERCENTAGE ROSETTE-FORMING T-LYMPHOCYTES IN BLOOD.

	SRBC's/lymphocyte				E-Rosettes[a]	
	≥1		≥2			
Samples	Wet	Automatic smearer	Wet	Automatic smearer	Wet	Automatic smearer
Normal volunteers						
1	55.0	56.2	44.0	43.3	42.0	40.6
2	67.5	65.7	59.9	58.6	56.4	51.7
3	50.7	62.2	44.2	54.6	41.9	50.1
4	58.3	76.7	47.3	61.9	45.1	55.8
5	74.6	62.1	67.6	56.8	65.0	52.7
6	73.2	71.8	67.0	63.4	65.4	59.6
7	71.6	71.4	59.6	62.4	57.3	58.8
8	84.4	78.7	81.0	69.9	79.2	66.9
9	81.6	76.6	77.6	72.0	72.8	68.0
10	62.9	64.0	58.1	61.0	54.3	58.8
Mean	68.0	68.5	60.6	60.4	57.9	56.3
±1 SD	11.2	7.6	13.0	8.1	12.8	8.1
SE	3.5	2.4	4.1	2.5	4.0	2.6
Patients						
MW (CLL)[b]	36.0	26.9	28.0	22.3	24.0	18.0
CS (CLL)	11.0	11.4	NA	6.4	2.0	4.7
CR (CLL)	28.2	28.1	21.2	25.7	19.5	24.9
AL (SS)[c]	72.4	72.8	66.4	66.0	64.0	59.6

E-Rosettes were defined as lymphocytes with 3 or more SRBC.

CLL, Chronic Lymphocytic Leukemia.

SS, Sézary syndrome; NA, not available.

taining the drop of blood at a constant selected speed against a glass spreader itioned at a fixed angle. Air-dried smears re fixed in absolute methanol for 5 min l stained with Wright Giemsa. Fiveidred lymphocytes were counted under it microscope.

n some instances, permanently fixed les of E-rosettes were prepared according he manual smearing technique (4).

he results were recorded as follows: lymcytes without SRBC and with 1, 2, and SRBC attached. Lymphocytes with 3 or re SRBC attached were considered to be osettes.

esults. In normal volunteers E-rosettes ged from 42 to 79% by hemocytometer from 40 to 68% by Hemaprep method ble I). The range of E-rosettes in pats with CLL was 11 to 36% by hemocyteter and 11.4 to 28% by Hemaprep thod (Table I). The E-rosettes from the ient with Sézary syndrome as obtained automatic smearing technique (59.6%) shown in Fig. 1 (magnification 186) and . 2 (750×).

The rosettes from manually prepared slides were not enumerated as there was considerable disruption of rosettes.

Discussion. Quantitation of B- and T-lymphocytes has proven to be of value in the study of immune diseases (5). E-Rosettes are commonly determined in the cell suspension using a hemocytometer. E-Rosettes in the normal human peripheral blood have ranged from 5 to 80% as reported by various investigators (2, 6, 7). Fragile nature of E-rosettes is one of the factors responsible for this wide range of difference. These nonimmune rosettes are known to be very sensitive to physical changes such as temperature, pH of the medium, incubation period, and trauma of manipulation during the preparation for counting (8–11).

A manual smearing technique has been described to prepare fixed and stained preparations of E-rosettes (4). In our hands considerable disruption of rosettes occurred during manual smearing, resulting in reduced number of E-rosettes as compared to the generally accepted hemocytometer method. The difference in the individual manual

FIG. 1. This photograph shows rosette-forming and nonrosette-forming lymphocytes from a smear made by automatic smearer. This preparation is from the blood lymphocytes of a patient with Sézary syndrome. (186×)

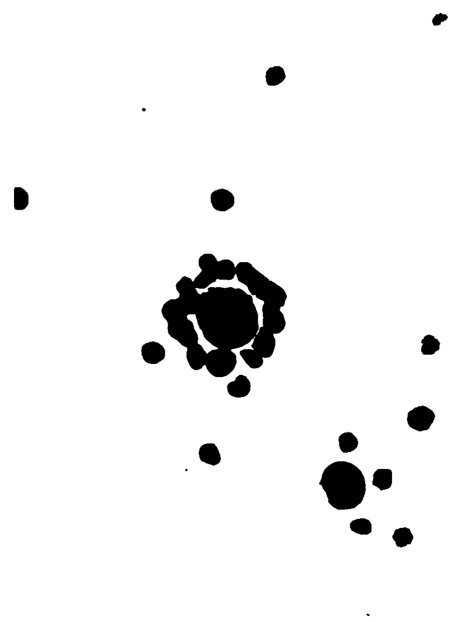

FIG. 2. Rosette-forming cell from the same preparation as shown in Fig. 1 at higher magnification (750×). Typical convoluted nucleus of Sézary cell is clearly identified.

smearing technique, angulation of the smearing slide, and the size of the drop of the suspension containing E-rosettes could each be contributing to the destruction of the rosettes. Cytocentrifugation, an alternate to manual smearing, may also produce changes in the apparent proportion of E-rosettes and was not recommended by a recent report from a World Health Organization sponsored workshop (12).

In order to eliminate the individual variations of manual smearing and the disruptive changes produced by manual or cytocentrifugation techniques, we investigated the use of Hemaprep for preparation of fixed smears of E-rosettes. The results obtained were reproducible and comparable to the hemocytometer method. The smears made with the automatic smearer showed no disruption of E-rosettes and the morphological features of the rosettes-forming cells could be clearly identified (Figs. 1 and 2).

Summary. Human T-lymphocytes were enumerated in 10 normal volunteers and 4 patients by a new method utilizing an automatic smearing instrument to prepare permanent fixed slides of E-rosettes. The results were compared to the commonly employed hemocytometer method.

Results obtained smearing instrument were similar to those obtained by the hemocytometer method with the added advantages of permanent record, convenience of quantitation, and precise morphological identification of rosette-forming cells. As opposed to well-preserved E-rosettes obtained by the automatic smearing instrument, hand-made smears showed disruption of E-rosettes.

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Ethanol-Prostaglandin Interactions in Contraction of Vascular Smooth Muscle[1]

(39391)

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Many studies involving prostaglandin (PG) molecules employ ethanol as a solvent. Collier and his co-workers have recently demonstrated that ethanol can stimulate biosynthesis of PGE and F compounds in rat stomach smooth muscle (1). Two preliminary reports suggest that this alcohol can affect excitability and reactivity of rat aortic and portal venous muscles *in vitro* (2, 3). In view of these diverse actions of ethanol, and its possible importance in studying PG molecules, we examined the effects of ethanol on contractions of arterial and venous muscle induced by PGE$_1$, A$_1$, and B$_1$ molecules.

Methods. Thoracic aortas and portal veins were obtained from male rats (Wistar strain, 275–425 g) after sacrifice by decapitation. The aortas were cut helically into vascular strips (1.5–2 mm in width by 25 mm in length) and the force of contractions were recorded *in vitro*, essentially as described previously (4). Ten-millimeter segments of portal veins were tied at both ends with sutures and the force of contractions were recorded *in vitro*, as described previously (5). Both aortic strips and portal vein segments were equilibrated for 2 hr in 20-ml muscle chambers containing Krebs–Ringer bicarbonate (KRB) solution (4) at 37° oxygenated continuously with 95% O_2–5% CO_2 (pH 7.4–7.5). The recording equipment was identical to that described previously (6). After the 2-hr incubation period the vascular segments were exposed to one of the three different PG compounds (E$_1$, A$_1$, or B$_1$) in concentrations which were either (i) above threshold; or (ii) required for eliciting 50% of a maximum PG contractile response. These respective responses were then repeated two more times, after washing and relaxation in KRB solution. The vascular segments were exposed to ethanol concentrations of 17, 170, and 430 m*M* for 5 min. (Preliminary experiments had shown there was no difference in effects when ethanol exposure was as long as 60 min.) All of the PG compounds were dissolved in phosphate buffer (pH 7.40–7.45) (7). The total volume of phosphate buffer used never exceed 0.2 ml. At least six different preparations were examined with each ethanol-PG dose combination. Significance of changes in tension were calculated by Student's *t* test. Tension values for the rat portal vein were calculated in a manner similar to that described previously (5, 8).

Results. Tables I and II demonstrate that a low concentration of ethanol (i.e., 17 m*M*) can markedly inhibit the contractile action of PGE$_1$ on both rat aortic strips and portal veins. Although lower concentrations of ethanol (i.e., down to 5 m*M*) can also attenuate PGE$_1$ contractions, in these two types of vascular muscle these effects were seen in only about 50% of the preparations examined. Higher concentrations of ethanol, i.e., 170–430 m*M*, produced greater inhibition of PGE$_1$ responses which appeared to be dose-related; the greater the ethanol concentrations, the less the PGE$_1$ response (Tables I and II). Although not shown, these ethanol-induced effects as well as those described below are completely reversible upon washing of the tissues in normal KRB solution.

In contrast to what is seen for PGE$_1$, preincubation of aortic strips with 17 m*M* ethanol enhances contractions induced by PGB$_1$ (Tables 1 and 2). Even high concentrations of ethanol, i.e., 170 m*M*, can still

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LE I. DIFFERENTIAL EFFECTS OF ETHANOL ON CONTRACTIONS INDUCED BY PROSTAGLANDINS IN RAT AORTIC STRIPS.

›mpound g/ml)	*N*	Control tension (mg ± SE)	Tension (mg) with ethanol (m*M*)		
			17	170	430
$_1$ (1)	8	572.8 ± 52.6	405 ± 36^a	166.6 ± 22^a	58.3 ± 12.6^a
$_1$ (0.25)	6	491.6 ± 84.7	925 ± 72.2^a	683.3 ± 42.3^a	133.3 ± 28.9^a
$_1$ (0.25)	8	825 ± 42.6	937.5 ± 20.5^a	200 ± 34^a	106.2 ± 38.2^a

ificantly different from control tension ($P < 0.02$).

.E II. DIFFERENTIAL EFFECTS OF ETHANOL ON CONTRACTIONS INDUCED BY PROSTAGLANDINS IN RAT PORTAL VEINS.

›mpound g/ml)	*N*	Control tension (mg ± SE)	Tension (mg) with ethanol (m*M*)		
			17	170	430
E_1 (1)	8	693.1 ± 23.1	531.2 ± 19.9^a	475 ± 34.6^a	43.7 ± 10.2^a
B_1 (1)	6	700 ± 25	858.3 ± 29.9^a	550 ± 22.6^a	91.6 ± 10.2^a
A_1 (1)	8	575 ± 28.3	750 ± 25^a	518 ± 31	300 ± 10^a

ficantly different from control tension ($P < 0.02$).

›otentiation of B_1 (Tables 1 and 2).
1 and 2 demonstrate that a low con-
ion of ethanol (e.g., 17 m*M*) can also
ate contractions induced by PGA_1.
er, unlike that seen with B_1, higher
trations of ethanol (i.e., 170 m*M*)
lly attenuate PGA_1 contractions (Ta-
and 2). Although not shown, the
ate buffer vehicle itself does not at-
(or enhance) baseline tension, am-
, or frequency of the spontaneous
ical responses of the vascular prepa-
, even when administered in volumes
three times that used with the doses
compounds.

ussion. The data presented here indi-
at low concentrations of ethanol can
ate or inhibit the contractile actions
compounds on at least two different
of vascular smooth muscles.[3] Al-
both inhibition and potentiation by
was seen with PGA_1 and PGB_1 com-
, only contractile inhibition was seen
e PGE_1 compound. The fact that the
ction of ethanol was demonstrated
ith PGE_1 and on both arterial and venous smooth muscle suggests to us that some part(s) of the structure of a prostaglandin molecule may be quite important in ethanol-PG interactions in contractions of vascular muscle.

Although ethanol is considered to be a CNS depressant (10), it can depolarize neurons (10, 11). In addition, it has been demonstrated that low concentrations of ethanol can increase activity of afferent nerves from primary and secondary endings in the muscle spindles and tendon organs of rat caudal muscle (12) as well as cause singly excited cat soleus motor nerve terminals to fire repetitively (13). Such reports suggest that ethanol may have both excitatory and depressant effects on certain excitable tissues. In addition, ethanol has been demonstrated to increase the frequency of miniature endplate potentials of rat phrenic nerve diaphragm preparations and to concomitantly increase the force of contraction (14).

In the latter study, Gage reported that ethanol increased the quantal content of the end-plate potential and the frequency of minature endplate potentials. Such actions were used to explain the resultant increase in the force of contraction (14). An effect of ethanol on the resting membrane potentials of the rat aorta and portal vein could explain the potentiation of PGB_1 and A_1 contractions.

A second possibility is that ethanol can stimulate endogenous synthesis of PG com-

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wing the procedure recommended by The
Co. for preparation of stock solutions, one
issolves the PG compounds in 100% ethanol
mg/ml (9). A dilution of 1 mg/ml to 1 μg/ml
ult in yielding approximately 17 m*M* ethanol,
r words, the concentration of ethanol which
ally affects the contractile actions of the sev-
ompounds used in the present study.

pounds (1). Since it has recently been demonstrated that synthesis of PG compounds can be stimulated in both arterial and venous smooth muscle by certain vasoactive substances (15), the latter hypothesis is plausible.

A third alternative hypothesis to explain ethanol enhancement of PG responses might be related to the effects of this alcohol on the intracellular availability of free, ionized calcium (Ca^{2+}). Ethanol has been demonstrated to affect the movement of Ca^{2+} in certain excitable tissues (16). Ethanol-induced enhanced uptake, or release of intracellular Ca^{2+} could result in a potentiation of PG contractions.

In this context, it is known that a wide concentration range of ethanol (i.e., 50–430 m*M*) can inhibit the contractile effect of Ca^{2+} in potassium-depolarized rat aortic strips and portal vein segments (2, 3, 17, 18). Since Ca^{2+} is undoubtedly the link between excitation and contraction for PG compounds in blood vessels (19, 20), high concentrations of ethanol could inhibit these contractions by simply reducing the availability of free, ionized Ca^{2+} (17, 18). Alternatively, one might be tempted to suggest that the inhibitory effects of ethanol on PG-induced contractions might be due to an affect of the alcohol on vascular smooth muscle cell metabolism and/or the contractile proteins. The present results, however, indicate that although different concentrations of ethanol can potentiate or inhibit PGA_1 or B_1 responses, the contractile responses elicited by PGE_1 are only inhibited. If ethanol exerted a general effect on metabolism and/or the contractile proteins, one might expect a particular concentration of the alcohol, e.g., 170 m*M*, to be inhibitory for all three PG compounds. The latter is, however, not the case.

Irrespective of the exact mechanism(s) by which ethanol enhances and attenuates PG contractions of aortic and venous smooth muscle, one must be cautious in the use of alcohol as a solvent for PG compounds when investigating vascular smooth muscle.

Summary. The present results, using isolated rat aortic strips and portal vein segments, demonstrate that ethanol (5–430 m*M*) can both enhance and attenuate the contractile actions of a variety of prostaglandin (PG) compounds on at least two different types of vascular smooth muscle. At the very least, the present findings question the use of ethanol as a solvent when investigating the contractile actions of PG molecules on smooth muscle.

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ɔmparative Effectiveness of Six Antiviral Agents in Herpes Simplex Type I Infection of Mice (39392)

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as previously been shown that several
al compounds, interferon, and in-
of interferon are effective in prevent-
erpes simplex virus, type 1, (HVH)
ons (1-7). The comparative efficacy
se agents has been difficult to evalu-
rtly because they generally have been
d under different experimental condi-
In addition combination therapy for
infection has not been thoroughly ex-
. The present study therefore was un-
en to compare under similar experi-
l conditions the therapeutic effective-
f six antiviral agents during systemic
infection in mice. Combinations of
of the drugs also were evaluated. The
tested were adenine arabinoside
-A) cytosine arabinoside (ARA-C),
idine (IDU), phosphonoacetic acid
), and the interferon inducer polyino-
olycytidylic acid (In.Cn). Particular
sis was placed on examining the tox-
f the various compounds and effec-
s against HVH-induced mortality.
erials and methods. Mice and virus.
type 1 strain VR_3, was originally ob-
from Dr. A. J. Nahmias and was
ed in primary rabbit kidney cells. The
nfectivity was determined by the mi-
que assay in BHK-21 cells. NIH Gen-
urpose Swiss male mice of 22-27 g
sed throughout the study. They were
ated ip with 2 LD_{50} ($10^{5.0}$ $TCID_{50}$) of
in 0.2 ml of Eagle's MEM medium
% fetal bovine serum. Most deaths in
mice occurred between the seventh
eventh days after infection. On Day 7
nfection histologic examination of the
of infected mice revealed multiple
areas of focal necrosis with polymorphonuclear cell infiltration: less pathologically severe lesions were usually found in the brains and lungs of HVH-infected mice. HVH was consistently isolated from the brains (10^3-10^6 PFU/g) but rarely from the livers of such mice.

Drugs. All drugs were administered ip. Drugs injected on the same day as virus were given 2 hr after the virus. ARA-A was obtained in powder form from the Parke Davis Co., and suspended in distilled water to a final concentration of 40 mg/ml. The ARA-A was not soluble in water, and therefore the fine particles of ARA-A were well suspended before the injection. IDU was obtained from Calbiochem and diluted with phosphate buffered saline at pH 7.2, to a final concentration of 40 mg/ml. ARA-C was obtained from the Upjohn Co. and diluted with sterile water to a concentration of 5 mg/ml. Double-stranded In.Cn was purchased from P. L. Biochemicals, and prepared as a 1.0 mg/ml solution in phosphate buffered saline, pH 7.6.

Interferon was prepared in mouse C-243 cells using Newcastle disease virus as described previously (8). The final concentration was 10^4 reference units per ml. Phosphonoacetic acid (PAA) was obtained from Abbott Laboratories and diluted with distilled water to a final concentration of 20 mg/ml.

Statistics. Significance of differences in mortality was determined using the chi-square test. Significance of differences in survival time was determined using the Mann-Whitney *U* test. Percent protection was calculated by the formula, mortality rate in virus controls minus rate in treated group divided by mortality rate in virus controls multiplied by 100.

Results. Comparison of ARA-A, ARA-C,

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served.

TABLE I. IDU, ARA-C, OR ARA-A TOXICITY AND PROTECTION AGAINST HSV IN MICE[a]

			Mortality			
			Drug only		Virus infected[b]	
Expt. no.	Drug	Dose (mg/kg)	Dead/total	Mortality (%)	Dead/total	Mortality (%)
1	ARA-C	100	5/10	50	15/15	100
	ARA-C	50	2/10	20	12/15	80
	ARA-C	25	0/10	0	13/15	87
	IDU	1000	9/10	90	15/15	100
	IDU	500	2/10	20	11/15	73
	IDU	250	0/10	0	9/13	70
	None				18/30	60
2	ARA-A	1000	0/10	0	2/15	13[c]
	ARA-A	500	2/10	20	0/15	0[c]
	IDU	250	1/10	10	11/15	73
	ARA-C	25	2/10	20	13/15	87
	None				24/30	80
3	ARA-A	1000	2/10	20	1/15	7[d]
	ARA-A	500	0/10	0	0/15	0[c]
	IDU	250	2/10	20	7/15	47
	ARA-C	25	0/10	0	11/14	79
	None				13/30	40
4	ARA-A	250	0/20	0	1/20	5
	ARA-C	15	0/15	0	12/15	80
	ARA-C	10	0/15	0	11/15	73
	ARA-C	5	0/15	0	11/15	73
	IDU	150	0/15	0	10/15	67
	IDU	100	0/15	0	9/15	60
	IDU	50	0/15	0	11/15	73
	None				20/30	67

[a] Treatment started 2 hr after virus and repeated daily for a total of five doses ip.

[b] Virus-infected mice were inoculated ip with 2 LD_{50} ($10^{5.0}$ $TCID_{50}$) of HSV.

[c] $P < 0.01$.

[d] $P < 0.05$.

and IDU for toxicity and protection (Table I). In these experiments treatment was started 2 hr after virus and repeated daily for a total of five doses ip. All three dosage schedules of ARA-A used (1000, 500, and 250 mg/kg/day ip) were highly effective in reducing mortality in HVH-infected mice. ARA-C and IDU both exhibited significant toxicity at the highest doses used as measured by deaths in the uninfected controls. At a dose of IDU without obvious toxicity (150 mg/kg/day or less), no antiviral effect of IDU could be demonstrated. Similarly, a nontoxic dose of ARA-C (15 mg/kg/day or less) was not protective; mice which received the relatively nontoxic dose of 25 mg/kg/day of ARA-C have a significantly decreased survival time when compared to untreated infected mice ($P < 0.01$). Thus, ARA-A was highly effective when begun 2 hr after HVH infection, while ARA-C and IDU over a wide range of doses were not effective under similar experimental conditions.

Toxicity of and protection against HVH in mice by PAA (Table II). It has been reported previously that PAA, at a dose of 200 mg/kg/day, is effective in preventing paralysis and death of mice when given systemically 24 or 72 hr after HVH infection (9). In the present study the same dose of PAA was nontoxic following systemic treatment of mice. However, as shown in Table II, no significant protection was noted. In similar experiments (not shown) there also was no protection even when mice were treated with PAA 24 hr before virus infection.

Postinfection treatment with ARA-A of HVH-infected mice. Experiments were per-

TABLE II. PAA TOXICITY AND PAA PROTECTION AGAINST HSV IN MICE.

Expt. no.	Drug	Dose (mg/kg)	Days of treatment	Mortality: Drug only: Dead/total	Mortality: Drug only: Mortality (%)	Mortality: Virus infected[a]: Dead/total	Mortality: Virus infected[a]: Mortality (%)	Mortality: Virus infected[a]: Survival time significance
1	PAA	200	3-8	1/10	10	14/15	93	0.7
	None					14/15	93	
2	PAA	200	1-6	ND[b]	ND	8/14	57	0.4
	None					10/15	67	
3	PAA	200	1-3	ND	ND	5/15	33	0.3
	None					9/15	60	

[a] 2 LD_{50} ($10^{5.0}$ $TCID_{50}$) of HSV injected ip on Day 0.

[b] Not done.

formed to determine how late in the course of HVH infection therapy with ARA-A could be initiated and still be effective (Fig. 1). Treatment with ARA-A (1000 mg/kg/day for 5 days) begun 2 or 3 days after virus inoculation was still highly effective in preventing deaths. Thus therapy with ARA-A was effective when begun as late as 3 days after experimental HVH infection.

Combination therapy against HVH infection in mice. Since by themselves ARA-A, interferon, and In.Cn (an inducer of interferon; (10)) have been shown to prevent HVH infection in mice, a series of experiments were performed to determine if combinations of these drugs, could provide even more protection than the drugs alone. In numerous experiments no mortality or obvious morbidity was noted in mice which received just In.Cn or interferon at the dose levels used in the above experiments. Similarly, administration of both In.Cn and interferon was without obvious toxicity in uninfected mice. Treatment with either In.Cn or interferon beginning on Day 3 did not result in significant reduction in mortality (Table III). Treatment with In.Cn was not continued beyond 2 days since hyporesponsiveness of continued interferon production occurs after 2 days of treatment (10). Treatment with interferon (10^4 units daily for 5 days) beginning on Days 3 and 4 gave erratic protection in preliminary experiments. Since many of the deaths in these experiments occurred after termination of interferon treatment, additional experiments were performed using 8 days of treatment. Slight but significant protection by interferon as measured by survival time occurred in two of two experiments (Table III) (Experiments 2 and 3), but mortality was not significantly reduced by interferon treatment. Combined In.Cn and interferon therapy resulted in both a decrease in mortality and a prolonged survival time. However, when the combined In.Cn and interferon therapy group was compared directly with the group which received only interferon,

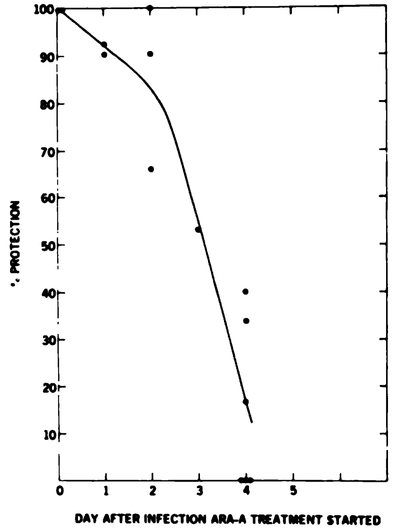

FIG. 1. Postinfection treatment with ARA-A (100 mg/kg/day ip for 5 days) of HVH-infected mice. Each dot represents percentage protection in an individual experiment when therapy was begun on the indicated day.

TABLE III. Therapy with Interferon and In.Cn against HSV Infection in Mice.

Expt. no.	Drug	Dose schedule	Mortality		
			Dead/total	Mortality (%)	Survival time significance
1	In.Cn	100 μg Days 3, 4	6/15	40	0.4
	In.Cn	50 μg Days 4, 5	7/15	47	1.0
	Interferon	10^4 units Days 3–7	11/15	73	0.5
	Interferon	10^4 units Day 4–8	9/15	60	0.9
	In.Cn + Interferon	100 μg Days 3,4			
		10^4 units Days 5–9	7/15	47	0.18
	None		8/15	53	
2	In.Cn	100 μg Days 3, 4	12/15	80	0.8
	Interferon	10^4 units Days 4–11	9/15	60	0.01
	In.Cn + Interferon	100 μg Days 3, 4			
		10^4 units Days 4–11	7/15	47	0.03
	None		12/15	80	
3	In.Cn	100 μg Days 3, 4	14/15	93	0.9
	Interferon	10^4 units Days 4–11	12/15	80	0.05
	In.Cn +	100 μg Days 3, 4			
	Interferon	10^4 units Days 4–11	7/15	47[a]	0.03
	None		14/15	93	

[a] $P < 0.05$.

the survival times and the decreased mortality were not significantly different.

It has recently been reported that ARA-A and human interferon are synergistic *in vitro* against HVH (11); the possibility was therefore suggested that combined therapy with interferon and ARA-A might be significantly more effective *in vivo* than therapy with either agent alone. In other experiments, not shown here, ARA-A was combined with either In.Cn (four experiments) or interferon (three experiments) in the therapy of this experimental HVH infection. The dosages of these drugs were: (a) ARA-A, 1000 mg/kg/day for 5 days beginning on Day 3 or 4; (b) In.Cn 50 or 100 μg/day for 2 days beginning on Day 3 or 4; (c) interferon 10^4 units/day for 5 or 8 days beginning on Day 3 or 4. The combinations were no more effective in decreasing mortality or prolonging survival time than the ARA-A alone. In fact the combination of ARA-A and In.Cn proved to be toxic, giving a mortality range of 33 to 90% in the uninfected controls. Even when the dosage of In.Cn was decreased to 12.5 μg daily for 2 days, in combination with ARA-A at 1000 mg/kg daily for 5 days, there was still high mortality in the uninfected controls. Only when the dose of ARA-A was reduced to 500 mg/kg/day for 5 days was there no mortality in the uninfected controls. When HVH-infected mice were treated with this nontoxic combination (500 mg/kg/day ARA-A and 100 μg In.Cn for 2 days and 50 μg In.Cn/day for 3 more days), no reduction in mortality or increase in survival time was observed.

Discussion. The present findings extend the previous demonstrations of efficacy of ARA-A by showing its clear superiority over the five other antiviral agents tested under the same conditions against HVH infection of mice. Interferon at the highest dose used was less protective than was ARA-A while ARA-C, IDU, PAA, and In.Cn had no antiviral effect in the present system. Of the several combinations of antiviral drugs tested, none was significantly more effective than either agent alone. Unexpectedly the protective and nontoxic 1000 mg/kg dose of ARA-A, when combined with nontoxic doses of In.Cn, resulted in a synergistic mortality of 33–90% in uninfected mice.

Similar to the present findings a number of previous studies of ARA-A have shown it to be effective in the treatment of HVH and vaccinia virus infections in mice, even when therapy with ARA-A was initiated as late as after infection (1, 4, 5, 7). In three of previous studies, treatment with -A appeared to be superior to that IDU when the two drugs were used in ame experiments (1, 4, 5). Particularly uraging has been the recent report of a le-blind trial which demonstrated the tiveness of systemic ARA-A in the nent of herpes simplex keratouveitis in (12).

has been somewhat more difficult to nstrate a significant protective effect ther ARA-C or IDU in experimental infections. In mice, ARA-C has been n to be effective only when HVH and were both given by intracerebral inoc- n (1, 13, 14). In an experimental infection in rats, which are relatively ant to the toxic effects of ARA-C, a ficial effect of ARA-C was observed when initiation of therapy was delayed days after virus inoculation (15). Simi- the present findings in mice, ARA-C oted to have a deleterious effect on ninated herpes zoster infection of man Reports such as these emphasize the sity of very careful evaluation of anti- drugs which have significant side ef- particularly immunosuppressive ef- Consistent with the present findings, ffect of IDU on mortality has been l in experimental HVH infection of or encephalitis and disseminated HVH ion in marmosets (17, 18), although beneficial effects were noted in guinea 19, 20).

contrast to our negative findings with , it has been noted to be effective in al experimental HVH infections in even when therapy was delayed 72 hr initiation of infection (9). Possible rea- or the differences in protection include of virus or mice and routes of adminis- n and dissemination of virus.

using combinations of different anti- agents which are effective through dif- mechanisms of action and which pos- liffering toxicities, it was hoped that effective therapy of this experimental ion could be obtained. This was partic- true since ARA-A and interferon een reported recently to be synergis- *vitro* against HVH (11). In fact, this sort of added effectiveness was not noted.

The superior therapeutic effectiveness of ARA-A may be related to its relative lack of toxicity. The deleterious effects of ARA-C during HVH infections in mice and man (16) emphasize the need for careful evaluation of potentially toxic drugs. Equal caution must be used in the evaluation of combinations of antiviral drugs, since unexpected toxicities may be noted when different drugs are combined. The observed combined toxicity of nontoxic doses of ARA-A and effective but nontoxic doses of In.Cn (as determined previously (23)) provides such an example.

Summary. The therapeutic effectiveness of six antiviral agents and certain combinations were evaluated under the same experimental conditions during systemic HVH infection of mice. The agents used were adenine arabinoside (ARA-A), cytosine arabinoside (ARA-C), idoxuridine (IDU), polyinosinic.polycytidylic acid (In.Cn), interferon, and phosphonoacetic acid (PAA). Treatment with ARA-A begun as late as 3 days after the virus infection was highly effective in preventing death. Interferon begun on Day 4 prolonged survival. In similar experiments, no antiviral effect of either IDU or PAA could be demonstrated, while therapy with ARA-C potentiated the infection. Of the drug combinations tested, none was more effective than the better agent alone. Unexpectedly a protective and nontoxic dose of ARA-A when combined with a nontoxic dose of In.Cn resulted in a synergistic mortality in uninfected mice.

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mmon Antigens of Herpes Simplex Virus 2, Associated Hamster Tumors, and Human Cervical Cancer[1] (39393)

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a previous communication (1), we re- ed on the demonstration of tumor-asso- d antigens (TAA) in sarcomas obtained direct inoculation of newborn hamsters vith herpes simplex virus type 2 (HSV- he antigens were detected in the sarco- as well as in the sera of tumor-bearing sters, by the immunoadsorption-in-gel od. The purpose of this investigation o determine the antigenic interrelation- between HSV-2, the HSV-2 associ- sarcomas and human cervical cancer, ed by other methods to HSV-2 (3).

terials and methods. Viruses. The MS SV-2 and the VR$_3$ strain of HSV-1 were agated in HEp-2 cells grown in Eagle's A with 2% fetal calf serum (FCS).

mors. The two HSV-2 associated ham- sarcomas, OT-1 and OT-11, studied r (1) were employed. For control pur- s, Para (defective SV40)-adenovirus 7) hamster tumor (obtained from F. Rapp, Hershey Medical Center) used. Cervical cancer biopsy tissues x) and a cervical cancer cell line (C4- ere obtained from Dr. E. Franklin, ry University School of Medicine, and . Auersberg, University of British Co- ia, Vancouver, respectively.

tigens and antisera. The methods for ration of tumor or normal tissue anti- and of rabbit hyperimmune sera (listed ble I) have been reported earlier (1).

rification of TAA and anti-TAA of OT- mors. The immunoadsorption-in-gel ique, described previously (1), was

ological methods. (i) Immunodiffusion Antisera to the virus or tumor preparations were reacted, after adsorption with appropriate antigens, with homologous and heterologous antigen preparations. The adsorption-in-gel procedure, previously employed (1), consisted essentially of placing the adsorbing antigen preparation in the central well, allowing it to diffuse for 2 hr at 37°, then replacing it with the antiserum to be adsorbed. The peripheral wells were filled with the homologous and heterologous test antigens, including the adsorbing antigens as controls. (ii) Passive hemagglutination (PHA) tests: The micro PHA procedure was employed, as described earlier (4). Each experiment was repeated at least three times.

Results. Table II presents the immunodiffusion results obtained when the antisera, adsorbed with appropriate antigens, were reacted with the homologous and heterologous antigens. Positive reactions indicate one or more precipitin lines. None of the adsorbed antisera reacted with the adsorbing antigens; neither did preimmunization sera react in these assays nor in any other serological test used in these studies.

Antiserum to HSV-2 reacted with HSV-2, HSV-1, OT-1, OT-11, and Ca Cx (Table II). A precipitin line of partial identity was noted between HSV-2, OT-1, OT-11, and Ca Cx (Fig. 1). When the anti-HSV-2 serum was adsorbed with HSV-2, no immunoprecipitin lines were formed with the previously positive homologous or heterologous antigens. Anti-HSV-1 serum reacted only with the two HSV antigens. No reaction appeared with homologous adsorption whereas adsorption with the heterologous HSV-2 antigen eliminated the reaction with HSV-2 only (Fig. 2).

Antisera to cervical cancer tissues and to C4-II cervical cancer cell line reacted with HSV-2, OT-11, Ca Cx, and C4-II with a

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[1] pported by an NSF Institutional Grant to Geor- te University, the Urban Life Center, NCI Grant 6806) and NCI Contract (NOICP 43393).

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TABLE I. ANTIGENS AND ANTISERA USED IN IMMUNODIFFUSION AND PASSIVE HEMAGGLUTINATION STUDIES.

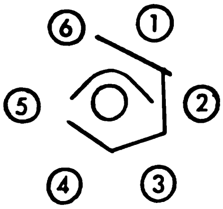
Test and/or adsorbing antigens	Homologous antisera prepared	Comments
Herpes simplex virus type 2 (HSV-2)	yes	MS strain
Herpes simplex virus type 1 (HSV-1)	yes	VR_3 strain
OT-1 tumor	yes	HSV-2 (MS strain)-associated hamster chondrosarcoma
OT-11 tumor	yes	HSV-2 (Pitts strain)-associated hamster hemangiosarcoma
OT-11 TAA	yes (purified)	Purified tumor-associated antigens of OT-11
Para-7 tumor	yes	Para (defective SV40)-adenovirus 7 hamster tumor
Normal hamster liver, muscle, and serum	no	Obtained from normal uninoculated inbred hamsters
C4-II cell culture	yes	Developed from human invasive cervical cancer tissue
Cervical cancer tissue	yes	Pooled cervical cancer tissues
Normal human liver and serum	no	Pools of 9 livers and of 12 human sera from noncancer individuals
Fetal calf serum	yes	Used for propagation of HSV-1, HSV-2, and C4-II
HEp-2 cells	yes	Used for propagation of HSV-1 and HSV-2

TABLE II. IMMUNODIFFUSION RESULTS WHEN VARIOUS ANTISERA, AFTER ADSORPTION WITH THE APPROPRIATE ANTIGEN(S), WERE REACTED WITH THE HOMOLOGOUS AND HETEROLOGOUS ANTIGENS.

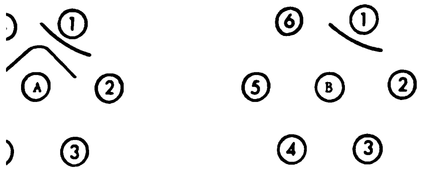
Anti-sera to:	Adsorbing antigens	Test antigens						
		HSV-2	HSV-1	OT-1	OT-11	Para-7	Ca Cx	C4-II
HSV-2	FCS[a] + HEp-2	+[b]	+	+	+	0	+	0
	HSV-2	0	0	0	0	0	0	
	HSV-1	+	0	+	+	0	0	
HSV-1	FCS + HEp-2	+	+	0	0	0	0	0
	HSV-1	0	0	0	0	0	0	
	HSV-2	0	+	0	0	0	0	
Ca Cx	NHuL[c] + NHuS[d]	+	0	+	+	0	+	+
	Ca Cx	0	0	0	0	0	0	
	OT-1	0	0	0	0	0	+	
	OT-11	0	0	0	0	0	+	
C4-II	FCS + NHuL	+	0	0	+	0	+	+
OT-1	NHL[e] + NHS[f]	0	0	+	+	+	+	0
	OT-1	0	0	0	0	0	0	0
	Para 7	0	0	+	+	0	+	0
	Ca Cx + NHL + NHS	0	0	+	+	+	0	
OT-11	NHS + NHL	0	0	+	+	+	+	0
	OT-11	0	0	0	0	0	0	0
	Para 7	0	0	+	+	0	+	0
	Ca Cx + NHL + NHS	0	0	+	+	+	0	
Para 7	NHS + NHL	0	0	+	+	+	0	0
	Para 7	0	0	0	0	0	0	0
	OT-1	0	0	0	0	+	0	0
	OT-11	0	0	0	0	+	0	0

[a] Fetal calf serum.
[b] + = one or more immunoprecipitin lines.
[c] Normal human liver.
[d] Normal human serum.
[e] Normal hamster liver.
[f] Normal hamster serum.

pattern of partial identity in one of the precipitin lines. Adsorption with Ca Cx removed homologous, as well as heterologous reactions, whereas adsorption of anti-Ca Cx serum with OT-1 or OT-11 did not eliminate reactivity with Ca Cx (Fig. 3).



. Immunodiffusion patterns: central well con- bit anti-HSV-2 serum adsorbed with HEp-2 FCS. The peripheral wells contain antigen ions of: (1) HSV-2; (2) OT-1; (3) OT-11; (4) 5) HEp-2 cells with FCS; (6) HSV-1.



. Immunodiffusion patterns: central wells con- bit anti-HSV-1 serum = (A) adsorbed with ells and FCS, and (B) adsorbed with HSV-2. ipheral wells contain antigen preparations of: -1; (2) OT-1; (3) OT-11; (4) Ca Cx; (5) HEp- nd FCS; (6) HSV-2.

isera to OT-1 and OT-11 reacted with and with the three hamster tumor ations, including Para-7. When these a were adsorbed with Para-7, two im- recipitin lines were noted with the ogous antigens, one of which showed identity with Ca Cx (Fig. 4). Adsorp- the anti-OT-1 and OT-11 sera with removed the reactivity with Ca Cx nti-Para-7 serum showed a common itin line with all three hamster tumor s, which was abolished after adsorp- ith either OT-1 or OT-11 (Fig. 5). TAA of OT-11 and antiserum to the ad been purified earlier (1) and these ations were tested by passive hemag- tion assays. As noted in Table III, 1:32 titers were obtained when anti- OT-11, HSV-2, and C4-II were re- with the purified TAA of OT-11, s antisera to HSV-1, HEp-2 cells, tal calf serum were nonreactive. purified antiserum to TAA of OT-11 sted with various antigen prepara- ositive reactions of 1:2 to 1:64 were d with HSV-2, OT-11, and C4-II, s no reaction was demonstrated with preparations of HSV-1, HEp-2 cells, FCS, and normal hamster liver (Table IV).

Discussion. Following the demonstration of tumor-associated antigens in sarcomas obtained after inoculation of HSV-2 in newborn hamsters (2), it was of interest to ascertain whether HSV-related antigens could be detected in these tumors. As noted in Table II, cross-reactivity was found between anti-HSV-2 sera and the HSV-2 associated hamster tumors (OT-1 and OT-11). Anti-HSV-2 serum also reacted by passive hemagglutination with the purified TAA of OT-11. Anti-HSV-1 serum did not react by either assay with any of the hamster tumors. The antisera to either OT-1 or OT-11 failed

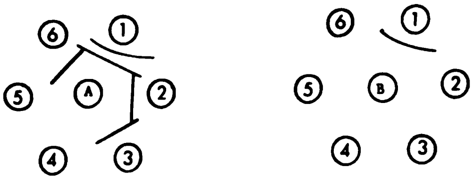


FIG. 3. Immunodiffusion patterns: central wells contain rabbit anti-Ca Cx serum = (A) adsorbed with NH$_u$L + NH$_u$S, and (B) adsorbed with OT-1 or OT-11. The peripheral wells contain antigen preparations of: (1) Ca Cx; (2) OT-11; (3) C4-II; (4) Para-7; (5) NH$_u$L + NH$_u$S; (6) HSV-2.

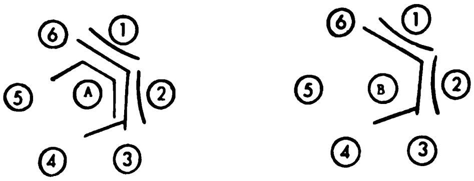


FIG. 4. Immunodiffusion patterns: central wells contain rabbit anti-OT-1 serum = (A) adsorbed with NHL + NHS, and (B) adsorbed with Para-7. The peripheral wells contain antigen preparations of: (1) OT-1; (2) OT-11; (3) Ca Cx; (4) C4-II; (5) NHL + NHS; (6) Para-7.

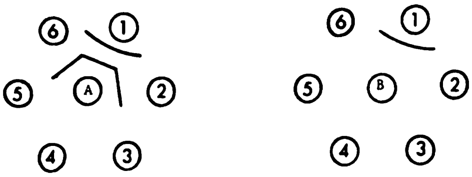


FIG. 5. Immunodiffusion patterns: central wells contain rabbit anti-Para-7 serum = (A) adsorbed with NHL + NHS, and (B) adsorbed with OT-1 or OT-11. The peripheral wells contain antigen preparations of: (1) Para-7; (2) OT-1; (3) Ca Cx; (4) C4-II; (5) NHL + NHS; (6) OT-11.

TABLE III. Passive Hemagglutination Titers Obtained with Purified TAA of OT-11.

Antisera to:	Reciprocal titers obtained with purified TAA of OT-11
OT-11	16
HSV-2	32
C4-II	8
HSV-1	<2
HEp-2	<2
FCS	<2

TABLE IV. Passive Hemagglutination Titers Obtained with Purified Antiserum to TAA of OT-11.

Antigens	Reciprocal titers obtained with antiserum to TAA of OT-11
OT-11	8
C4-II	64
HSV-2	2
HSV-1	<2
HEp-2	<2
FCS	<2
NHL	<2

to react in immunodiffusion tests with HSV-2, but a very weak reaction was obtained between the purified anti-TAA of OT-11 and HSV-2 in the PHA test (Table IV). The anti-HSV-2 serum failed to react with the Para-7 hamster tumor. Adsorption of the anti-HSV-2 serum with HSV-2 removed homologous reactivity, as well as heterologous reactivity with the HSV-2 associated tumors, implying the presence of common antigens between HSV-2, OT-1, and OT-11.

The hyperimmune rabbit anti-HSV-2, but not the anti-HSV-1, serum also reacted with cervical cancer tissue antigens (Ca Cs). The converse was also observed, i.e., that anti-Ca Cx serum reacted with HSV-2, but not with HSV-1. Adsorption of the anti-HSV-2 serum with HSV-2 eliminated the reaction with Ca Cx, suggesting that the common antigen may be specific to HSV-2. Antiserum to Ca Cx reacted with the HSV-2 associated hamster tumors and C4-II cervical cancer cell line, but not with the Para-7 hamster tumor. Absorption of anti-Ca Cx serum with the homologous antigens removed both homologous and heterologous reactivity. On the other hand, adsorption of the anti-Ca Cx serum with OT-1 or OT-11 did not abolish reactivity with Ca Cx. Anti-OT-1 or OT-11 sera adsorbed with Ca Cx still retained their reactivity with the hamster tumor preparations, suggesting that both common and specific antigens are present in the human and hamster tumors. It is likely that these common and specific antigens are different from those found between the HSV-2-associated hamster tumors and the non-HSV-2 related Para-7 tumor. Common reactivity between other HSV-related hamster tumors and Para-7 have also been found by other workers using other serologic tests (5). The antigen shared by the HSV-2 and the Para-7 associated hamster tumors may be explained by the possible existence of a fetal antigen. Such antigen may appear in the neoplasms as result of derepression of the gene in the malignant cell with reappearance of the embryonic antigen. Indeed, the presence of such antigen in SV_{40}-induced tumors in hamsters has been previously reported (6, 7). Attempts to elucidate the nature of the common antigen will be investigated in the near future.

Reactivity of anti-TAA of OT-11 with the C4-II cervical cancer cell line was only demonstrated by passive hemagglutination. The anti-OT-11 serum failed to react by immunoprecipitation with C4-II. Unfortunately, further tests were not possible with C4-II as the cell line was lost.

The above results indicate antigenic relationships between HSV-2, the HSV-2 associated hamster sarcomas and cervical cancer. Antigenic relationships between HSV-2 and the HSV-2 associated hamster tumors were suggested earlier by anticomplement immunofluorescent (ACIF) tests (8) which also pointed to a relation between HSV-2 and cervical cancer. Using different serological tests, other workers have also found antigenic relationships between this virus and human cancer (9, 10). Further work is in progress to characterize the antigens common to the virus and the hamster or human tumors.

Summary. Using the immunodiffusion-adsorption-in-gel and/or the passive hemagglutination techniques, an antigenic relationship has been found between Herpes simplex virus type 2 (HSV-2), cervical cancer tissue antigen preparations, a cell culture derived from cervical cancer, and two

HSV-2 associated hamster sarcomas. No cross-reactive antigens were detected between HSV-1, and the HSV-2 related hamster sarcomas or with human cervical cancer.

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Some β-Adrenergic Responses to Administration of *d,l*-Isoproterenol to Turkeys[1] (39394)

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Administration of the β-adrenergic agonist, isoproterenol, to rats and other mammals induces copious drinking and antidiuresis (1-7). The objective of the experiments described below was to determine whether isoproterenol also stimulated the same responses in a bird, viz, the turkey.

Methods. Experiment 1. Effect of isoproterenol on water intake of turkeys. Sixteen mature female turkeys (broad-breasted white) weighting 7.7 to 8.4 kg were used. The birds were divided equally into two groups. Group 1 served as the control group and was injected sc in the neck with 0.5 ml of isotonic saline. Group 2 was injected sc in the neck with 50 μg of *d,l*-isoproterenol sulfate dihydrate[2]/kg. The dose of isoproterenol was calculated as the salt, dissolved in saline, and 0.5 ml was injected. A watering device[3] from which the turkeys had been watered since 3 weeks of age was used to measure water intake at hourly intervals for 2 hr. A preliminary study showed that the turkeys would not drink from a watering device with which they were not familiar during the 2-hr test period. Intake of water was determined by weighing the water containers initially and at hourly intervals after administration of isoproterenol. Each turkey was kept separately in a pen 7-ft square. The floor was covered with peanut shells and the watering device was in a corner of each pen. During this study, it was observed that the birds treated with isoproterenol excreted a watery urine, a condition never observed in turkeys under normal circumstances, since these animals excrete a semisolid urine with their feces. A separate study was therefore made to determine whether drinking occurred prior to excretion of the watery urine or vice versa. Six control and six treated turkeys were used. The experiment was carried out as above except that a control and a treated turkey were observed continuously by one individual and notations were made of the times that drinking and urination occurred in each bird during the 2 hr following administration of 50 μg of *d,l*-isoproterenol/kg of body weight, sc.

Experiment 2. Effect of isoproterenol on urine excretion in turkeys. In this study an attempt was made to determine the effect of acute administration of isoproterenol on urine excretion in restrained turkeys.

Three mature female broad-breasted white turkeys weighing 5.0 to 7.1 kg were used. The turkeys were restrained in dorsal recumbency because it was found that they usually lay quietly throughout the 2-hr experiment when their heads were covered with a towel. Before beginning the experiment, each turkey was given an isotonic saline enema to cleanse the cloaca and lower gut of urine and feces. Completeness of the procedure was determined visually with the aid of canine vaginal speculum. Urine was collected continuously in a filtering flask trap by aspiration from the cloaca with a vacuum pump. Urine volume was measured every 30 min throughout the experiment. After an initial 30-min control period, each turkey was injected sc with 200 μg of *d,l*-isoproterenol sulfate dihydrate/kg body weight. The dose was calculated as the salt.

Experiment 3. Other β-adrenergic responses to administration of isoproterenol in turkeys. Five broad-breasted white turkeys, two male and three female weighing from 5.2 to 7.6 kg were used. The three female turkeys were also used in Experiment 2. In the first study, skin temperature of the left

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[1] Supported by Grants No. HL-14526-04 and No. HL-17909 from the National Heart and Lung Institute.

[2] *d,l*-Isoproterenol sulfate dihydrate, Aldrich Chemical Co., Milwaukee, Wisconsin.

[3] Little Giant Fountain Bowl #2508, Miller Mfg. Co., South St. Paul, Minnesota.

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f the birds was measured while they restrained on a table. A Yellow s Instruments Co. fast-response ther- probe #423 was taped to the skin of gh of each bird and temperature read- ere made at 1-min intervals during a control period and for 44 min after stration of isoproterenol (200 μg of roterenol sulfate dihydrate/kg sc). se was calculated as the salt. Mean nperature of the room during this vas 25.8°.

e second study, heart rate was re- by means of needle electrodes im- d in the breast muscles of eight re- ed turkeys and recorded on a Bio Systems polygraph. At the end of in control period, four treated tur- hree female and one male) were ad- red 200 μg of *d,l*-isoproterenol sul- ydrate/kg sc in the neck region. The as calculated as the salt. Four control turkeys not used in previous experi- were administered the saline vehicle ame time. Measurement of heart rate ed for 70-90 min following treat-

weeks later, four turkeys were ad- red 200 μg of *d,l*-isoproterenol sul- ydrate/kg as the salt and killed 1 hr An additional bird was administered instead of isoproterenol and killed 1 r. The kidneys from each bird were n 10% neutral formalin and embed- paraffin. Tissue sections were cut at 5 d stained with hematoxylin and eosin. stical analysis of the data included an s of variance, and both one- (Experi-) and two-tailed Student's *t* tests (Ex- nts 1 and 3) (8).

lts. Experiment 1.

inistration of isoproterenol to tur- creased their water intake in a fashion similar to that reported for mammals (Table I). Water intake of treated turkeys was more than fivefold that of controls during the first hour of treatment ($P < 0.05$). During the second hour after treatment with isoproterenol, water intake was significantly ($P < 0.01$) greater than that of saline-treated controls. The cumulative 2-hr water intake of the treated group was also significantly ($P < 0.01$) greater than that of the control group.

In a study separate from that described above, four of six turkeys administered isoproterenol urinated prior to drinking. The time between urination and drinking varied from 1 to 63 minutes. A third turkey drank 5 min after urination while the fourth drank 21 min after urination. In the case of the remaining two turkeys, drinking occurred 8 and 11 min prior to urination. Turkeys treated with saline were never observed to excrete a fluid urine.

Experiment 2. Urine excretion rate increased at each 30-min interval after administration of isoproterenol although the increases were not significant (Table II). In contrast, the change in rate of urine excretion from the preisoproterenol control period increased significantly ($P < 0.05$) at 60

TABLE II. RATE OF URINE EXCRETION IN THREE TURKEYS AFTER SC ADMINISTRATION OF 200 μg *d,l*-ISOPROTERENOL SULFATE DIHYDRATE/kg BODY WEIGHT

Postinjection time (min)	Urine excretion (ml/30 min)	Δ Urine excretion (ml/30 min)
0	38.3 ± 6.1*	—
30	74.7 ± 23.5	38.8 ± 22.5
60	126.0 ± 40.3	87.7 ± 38.7**
90	154.7 ± 56.0	116.3 ± 53.4**

* One standard error of mean.

** Significantly different from 0 time ($P < 0.05$); one-tailed Student's *t* test.

BLE I. EFFECT OF ADMINISTRATION OF *d,l*-ISOPROTERENOL SULFATE DIHYDRATE (50 μg/kg BODY WEIGHT) ON WATER INTAKE OF TURKEYS DURING 2 HR AFTER DRUG TREATMENT

		Water intake during:		
rimental group	Number of turkeys	1st hr	2nd hr	(1 + 2 hr)
rol	8	10.5 ± 4.5*	18.9 ± 7.9	29.4 ± 11.5
oterenol tested	8	56.4 ± 15.6**	105.5 ± 21.7***	161.9 ± 27.6***

standard error of mean.

nificantly different from control group ($P < 0.05$).

gnificantly different from control group ($P < 0.001$).

and 90 min after administration of isoproterenol. It should be pointed out that urine excretion rate found here does not reflect urine excretion rate in the noncatheterized bird whose cloaca normally reabsorbs the water brought to it.

Experiment 3. Administration of 200 μg *d,l*-isoproterenol/kg sc to turkeys increased the temperature of the skin of the left thigh. Maximal levels were achieved within 40 to 60 min after drug administration. In contrast, administration of saline had no effect. Representative results for three individual turkeys are shown in Fig. 1. In addition to increasing skin temperature of the thigh. administration of isoproterenol was also accompanied by more than a doubling of the respiratory rate (Fig. 1).

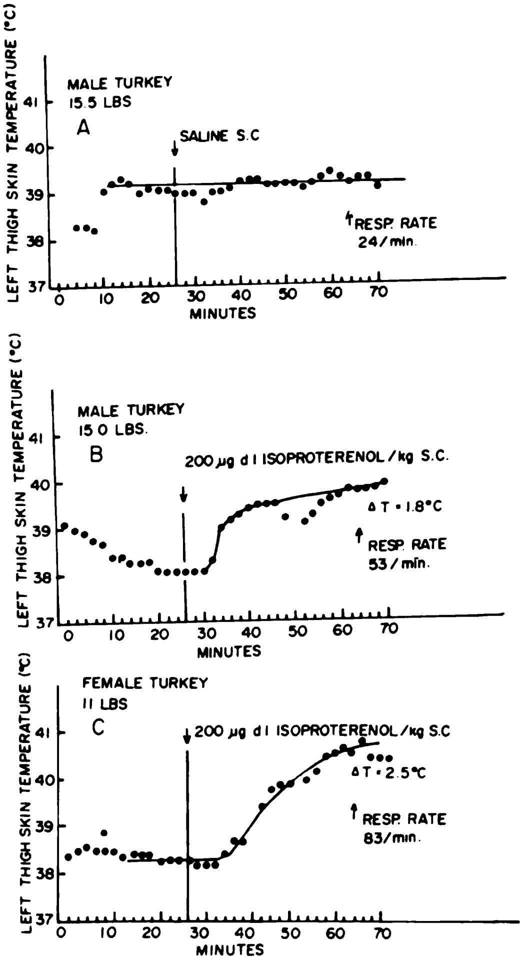

FIG. 1. Effect of sc administration of either saline (A) or *d,l*-isoproterenol sulfate dihydrate (200 μg/kg body wt) (B,C) on skin temperature of left thigh of turkeys. Respiration rate measured 38 min after treatment is also given in the figure.

Heart rate increased from levels of 170 to 270 beats per minute approximately 25 min after administration of 200 μg of *d,l*-isoproterenol/kg sc (Fig. 2). Control turkeys administered saline showed a reduction in heart rate from levels of 165 to approximately 155 beats per minute, 25 min after injection. Heart rate of the treated turkeys had not returned to control level 75 min after administration of isoproterenol.

Histological examination of the kidneys revealed that glomerular and peritubular capillaries were widely dilated in turkeys killed 1 hr after administration of isoproterenol (Fig. 3C). These dilations were not seen in the turkey treated with saline (Fig. 3A).

Discussion. This is the first report of drinking accompanying administration of isoproterenol to a bird. The drinking re-

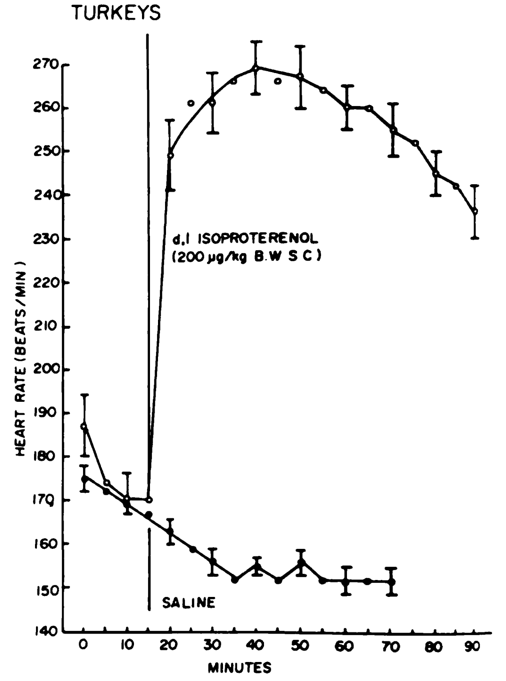

FIG. 2. Effect of sc administration of either saline or *d,l*-isoproterenol sulfate dihydrate (200 μg/kg body wt) on heart rate of turkeys. Treatment was initiated at the end of a 15-min control period. One standard error is set off at each mean.

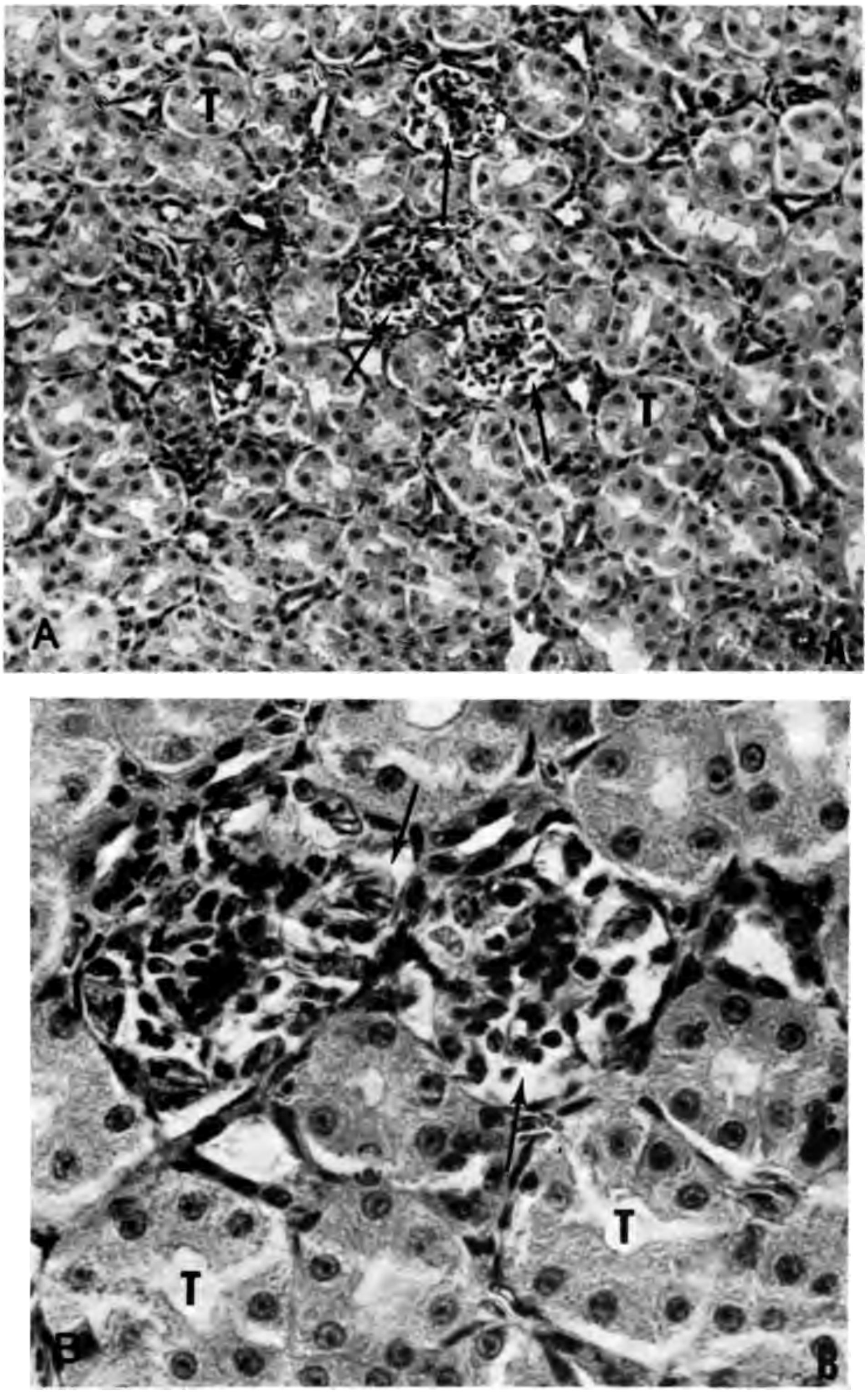

FIG. 3. The effect of a single injection of saline (A and B) or isoproterenol (C and D) on the histological appearance of the kidney of turkeys. (A) The glomeruli (arrows) are compact and tubules (T) are not dilated. H + E stain, × 250. (B) H + E stain, × 1300. (C) The glomeruli are swollen and their capillaries (arrows) dilated. Tubules (T) and peritubular capillaries (C) are also dilated. H + E stain, × 250. (D) H + E stain, × 1300.

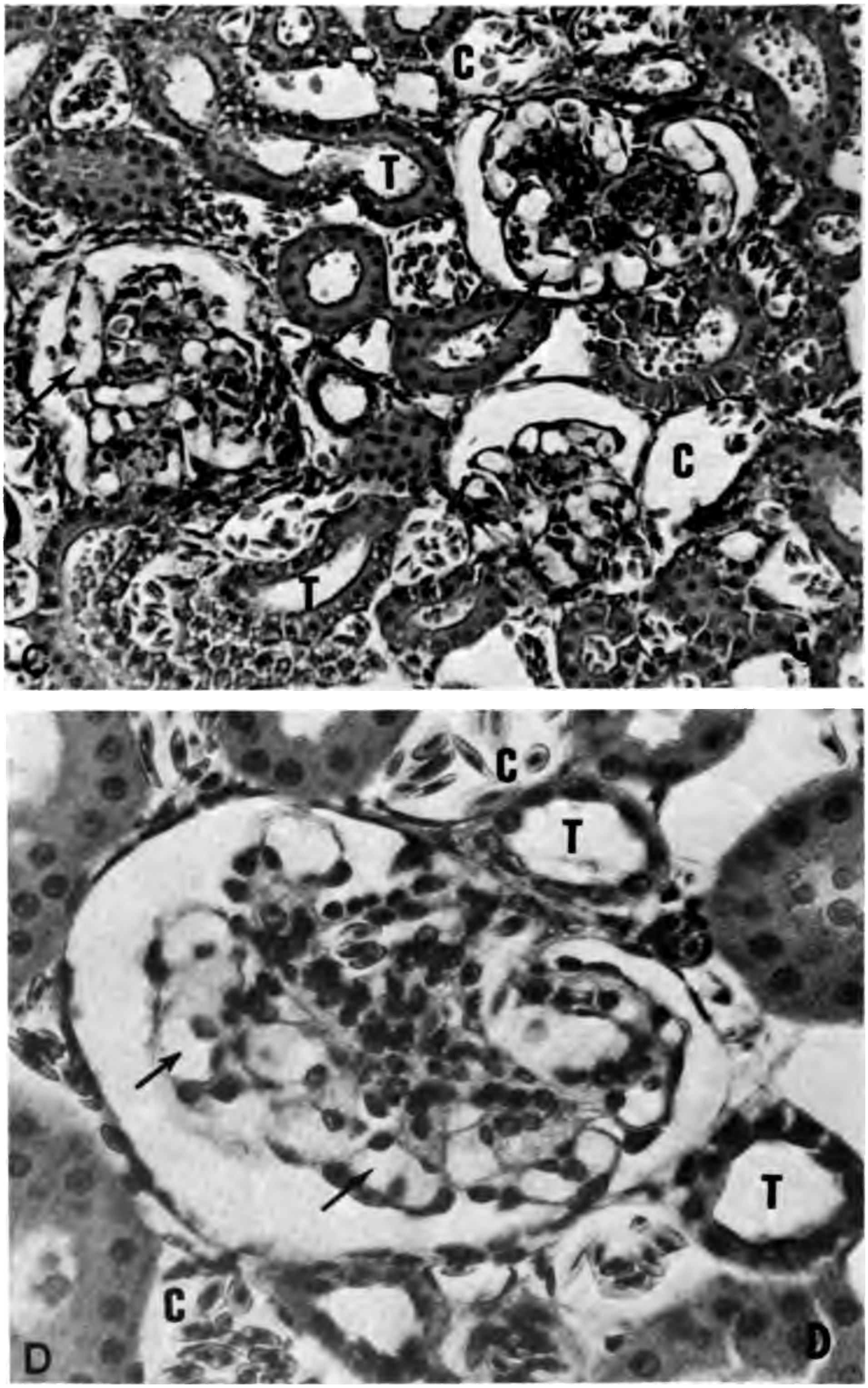

FIG. 3. *Continued*

: was similar in character to that ob-
l in mammals (1-5). However, the re-
: of the turkey appeared to differ from
f mammals in that administration of
terenol was accompanied by a fluid
excretion. This is a rare occurrence in
and contrasts sharply with the promi-
ntidiuresis accompanying administra-
f isoproterenol to mammals (3, 6, 7).
sibility exists that drinking may be
ated indirectly by the increased urine
ion since four of six turkeys studied
ed a fluid urine prior to drinking.
ver, two other turkeys drank prior to
ion.

innot be stated with certainty whether
creased urine excretion accompanying
istration of isoproterenol was related
to changes in renal hemodynamics or
eduction in antidiuretic hormone se-
n. An increase in glomerular filtration
id filtration fraction could account for
uresis as could changes in tubular
orption of electrolytes and water.
r *et al.* (6) and Berl *et al.* (7) have
that the antidiuretic effect of isopro-
l in the dog is not related to effects on
dney but to an increase in the rate of
on of antidiuretic hormone. The pos-
that isoproterenol may have opposite
in turkeys requires additional study,
s the dilation of glomerular capillaries
d by this drug (Fig. 3C).

view of the increased urine excretion
panying administration of isoprotere-
turkeys, it seemed important to study
ect of isoproterenol on some other β-
ergic responses to determine whether
iffered from those customarily seen in
nals. The studies revealed that in-
s in skin temperature (Fig. 1) and
ate (Fig. 2) in response to isoprotere-
re similar to those reported for mam-
9-11).

mary. Acute administration of *d,l*-
terenol (50 μg/kg body wt, sc) to tur-
vas accompanied by a significant increase in water intake which occurred within 1 h after treatment. This dose of *d,l*-isoproterenol also induced a copious, watery urine excretion. Acute administration of *d,l*-isoproterenol (200 μg/kg body wt, sc) increased the temperature of the skin of the left thigh within 10 min after treatment. Skin temperature reached maximal levels within 40 to 60 min after treatment. Heart rate of turkeys administered *d,l*-isoproterenol (200 μg/kg body wt, sc) increased approximately 100 beats per minute within 25 min after treatment. With the exception of the diuresis, the turkey responds to administration of isoproterenol in the same fashion as the mammal.

We thank Miss Charlotte Wright for drawing the figures, and Mr. T. W. Carlisle for technical assistance.

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Fetal Lymphoid Tissues: Increase in Antigen-Binding Cells Induced *in Vitro* by Antigen, LPS, and Aggregated IgG[1] (39395)

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Previous work has shown that by the tenth day of gestation, embryonic tissues involved in the ontogenesis of the mouse immune system (1, 2) contain small numbers of antigen-binding cells (ABC), specific for several antigenic determinants (3). Cells from these tissues, like those from adult lymphoid tissues, respond to antigen *in vitro* with proliferation (4), an increase in the number of cells bearing IgM, IgG, and IgA on their membranes (4, 5), and with the production of small quantities of specific antibody (4, 6).

Presented here are observations showing that when mouse fetal liver cells were cultured with antigen, specific ABC were formed and the number produced was increased if lipopolysaccharide (LPS) or aggregated human IgG had been added to the cultures; despite this increase in ABC there was no detectable increase in the number of antibody-secreting cells (plaque-forming cells, PFC). By contrast, LPS and aggregated IgG had no significant effect on the numbers of ABC in antigen-stimulated cultures of adult spleen cells, but they did have profound effects on the numbers of PFC generated in these cultures. These findings suggest that the appearance of specific antibody on the membranes of B cells or their precursors and the ability to secrete significant quantities of this antibody represent separate steps in the maturation of B cells.

Materials and methods. Tissue donors. Fetal liver and adult spleen and thymus cells were obtained from CBA/J mice (Jackson Laboratories, Bar Harbor, Maine). Embryonic age was determined by considering the day the vaginal plug was observed as day zero.

Culture conditions. Cells from adult mice were dissociated, washed, and added to the cultures described below at a concentration of 5 × 10^6/ml (4). Fetal liver cells from a single pregnancy were pooled; the cells were dissociated and added to cultures at concentrations of 1-to-10 × 10^6/ml. The medium was Eagle's S-MEM, with 10% fetal calf serum, L-glutamine, and penicillin-streptomycin (100 units and 100 μg/ml, respectively).

Primary responses to sheep red cells (SRBC) were elicited as described previously (4). Briefly, an underlay of washed SRBC (2%) in 1.5 ml of agar (0.25%) was placed in 35 × 10-mm tissue culture dishes (Falcon Plastics, Oxnard, Calif.) and 4 ml of cell suspension was added after the agar had hardened. Where indicated, heat-aggregated human IgG (63° for 20 min, 200 μg/ml; Miles Laboratories, Kankakee, Ill.) or LPS *Escherichia coli* B5 (5 μg/ml; Difco, Detroit Mich.) was added, and the cells were cultured for 3 days at 37° in 5% CO_2 in air. At this point the cells were removed from the plates, washed three times in a Ca- and Mg-free, buffered salt solution (PBS), and resuspended at a concentration of 25 × 10^6/ml. The SRBC-agar underlay were processed for areas of complement-dependent hemolysis (hemolytic foci; (4)). All cultures were done in triplicate. The aggregated human IgG did not produce agglutination of SRBC when tested over a wide range of concentrations.

SRBC-rosettes (ABC). The detection of cells specifically binding SRBC (ABC) was performed by standard methods (7, 8; cells having Fc and complement receptors were also determined but the results are not reported here). Briefly, 0.2 ml of cell suspension was added to 0.5 ml of PBS, and 0.1 ml of washed SRBC (1%). The cells were gently centrifuged, carefully resuspended, and examined in a hemocytometer under 400× magnification. A minimum of 2000

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d cells were scanned. The specific-
BC formation was demonstrated by
; cells with and without SRBC and
/ith SRBC and horse RBC, and vice
nlike human T cells, mouse cells do
ı nonspecific rosettes with SRBC.

se the addition of LPS or aggre-
;G to cultures often produced an
or decrease in proliferation, in each
ent the frequencies of ABC in cul-
ıtaining these agents were corrected
/s: observed frequency × total cells
:d: total cells recovered from cul-
ıtaining only SRBC. The correction
(mean and range) were (a) adult
LPS, 1.3 (0.75–2.0); IgG, 0.9
.1), (b) fetal liver: LPS, 0.96 (0.7–
J, 1.3 (0.9–2.2). The results from
riments were pooled and analyzed
s of Student's *t* test.

In six experiments the cells re-
from the cultures were assayed for
FC by a slide modification of the
:hnique (4).

nofluorescent studies. Cells ob-
rom cultures and SRBC-rosettes
amined for membrane-bound IgM
ıdirect immunofluorescent method
preparations. The anti-IgM serum
specifically with the heavy chain of
gM, and neither antisera cross-re-
th fetal calf serum or human IgG
Laboratories, Springfield, Va.).
ıymocytes did not bind either anti-

ɛ. When tested before culture very
; from adult spleen-bound SRBC
.4%, data not shown). After 3 days
re with SRBC, the frequency of ABC either did not change or, at most, increased threefold (Table I). The addition of LPS or aggregated IgG to the cultures did not consistently or significantly alter the frequency of SRBC-ABC; on the other hand, the numbers of hemolytic foci and PFC produced in these cultures were increased by LPS and decreased by IgG to significant degrees (Table II). Membrane-bound IgM was easily detected by immunofluorescent studies on SRBC-ABC taken from cultures of adult spleen, and the intensity of staining was only modestly, if at all, increased on cells taken from cultures containing LPS or IgG.

By contrast, no SRBC-ABC were detected with certainty among fetal liver cells before culture (i.e., <1:10,000); but after 3 days *in vitro* the numbers of specific SRBC-ABC significantly exceeded those recovered from cultures of adult spleen (Table I). The addition of LPS or aggregated IgG to these cultures resulted in further increases in the formation of ABC. More cells were recovered from cultures containing IgG than from the controls (up to 220%), but LPS had no significant effect on the number of cells recovered. Liver cells cultured with SRBC formed rosettes with SRBC but not with horse RBC, and vice versa.

As was the case with adult spleen cells, IgM was readily detected on the membranes of SRBC-ABC taken from cultures of fetal liver, but, in contrast, the presence of LPS or IgG in the medium resulted in a dramatic increase in the intensity of staining. However, neither LPS nor IgG significantly altered the number of PFC recovered from cultures of fetal tissues (Table II). As previ-

E I. FREQUENCY OF SPECIFIC ANTIGEN-BINDING CELLS AMONG FETAL LIVER, ADULT SPLEEN AND
HYMUS CULTURED FOR 3 DAYS WITH SRBC IN THE PRESENCE OR ABSENCE OF AGGREGATED HUMAN
IgG (200 μg/ml) OR LPS (5 μg/ml)

:o	Percentage of cells binding SRBC (± SD)[a]							
	Age of gestation						Adult spleen	Adult thymus
	9–10	11–12	13–14	15–16	17–18	19–20		
	0.2 ± 0.1	0.3 ± 0.1	1.1 ± 0.3	1.7 ± 0.5	1.9 ± 0.6	0.9 ± 0.4	0.7 ± 0.6[c]	<0.001
G		0.5 ± 0.1	2.1 ± 0.8[b]	3.4 ± 0.6[b]	3.6 ± 1.1[b]	1.7 ± 0.9	0.8 ± 0.7[c]	<0.001
			1.6 ± 0.2	2.7 ± 0.4[b]	2.2 ± 0.5	1.5 ± 0.3	0.5 ± 0.6[c]	<0.001

ta have been compiled from 19 separate experiments, and in each experiment the frequencies of ABC in cultures containing aggregated
ve been corrected as follows: observed frequency × total cells recovered: total cells recovered from cultures containing only SRBC.
(Student's *t* test), when compared to the number of ABC among cells cultured with SRBC only.
, when compared to values obtained with fetal liver cells from 13- to 20-day embryos cultured under the same conditions.

TABLE II. DEVELOPMENT OF HEMOLYTIC FOCI AND PFC BY ADULT SPLEEN AND FETAL LIVER CELLS CULTURED WITH SRBC IN THE PRESENCE OR ABSENCE OF AGGREGATED HUMAN IgG (200 μg/ml) OR LPS (5 μg/ml)[a]

Source of cells	SRBC in culture	Agent added	Number per 10^6 cells (± SD)	
			Hemolytic foci	PFC
Adult spleen	No		0	0
	Yes		12.2 ± 1.2	117 ± 10.4
	Yes	Agg.IgG	3.8 ± 0.9	19 ± 3.5
	Yes	LPS	16.7 ± 2.3	245 ± 30.6
Fetal liver (14 to 18 day)[b]	No		0	0
	Yes		0[c]	16.2 ± 5.2
	Yes	Agg.IgG	0[c]	12.1 ± 6.1
	Yes	LPS	0[c]	19.7 ± 3.4

[a] These data have been compiled from six experiments.

[b] Age of gestation.

[c] The agar in these plates contained from 4 to 10 defects approximately 1 mm in diameter that could represent complement-dependent hemolysis and/or enzymatic damage from overlying cells.

ously reported (4) the hemolytic plaques formed by fetal cells were small and few, but they were demonstrated to be specific for SRBC (i.e., no response against horse RBC). In addition, the plaques were complement dependent, and they could be completely inhibited by adding anti-IgM serum (1:40) or sodium azide (0.1%) to the slides. No hemolytic foci were identified with certainty on the agar underlay.

Discussion. Under the experimental conditions described here, when cells from adult mouse spleen were cultured with SRBC the frequency of specific ABC increased little, if at all; the addition of LPS or aggregated IgG to these cultures did not alter this. On the other hand, LPS increased and IgG decreased significantly the numbers of antibody-secreting cells recovered from these cultures. By contrast, under the same conditions, fetal lymphoid precursors produced relatively large numbers of ABC and this was further increased by the addition of LPS or IgG. Nevertheless, despite evidence from immunofluorescent studies of increased IgM on the membranes of ABC taken from cultures containing LPS or IgG (9, 10), these agents did not alter the number of antibody-secreting cells demonstrable in cultures of fetal tissue. Taken together, these findings suggest that the appearance of specific antibody on the membranes of B cells or their precursors and the ability to secrete significant quantities of this antibody represent separate steps in the maturation of B cells (1).

The increase in ABC induced by aggregated IgG and LPS in cultures of antigen-stimulated fetal liver cells was unexpected, and the mechanisms of action remain unclear. In these and in previous experiments (12) the addition of aggregated IgG to cultures of adult spleen cells resulted in no significant change in the frequency of ABC, but in up to 95% inhibition of PFC and of the binding of antigen-antibody complexes. While this suggests that the Fc receptor on B cells may be involved in the inhibition of PFC by IgG (13), it is not clear that the same mechanism is involved in modulating the frequency of ABC. The mode of action of LPS on fetal cells is even more obscure; it does not appear to be mitogenic for B cell precursors (14), and this suggests the possibility that its effect on ABC may be indirect.

Summary. When adult spleen cells were cultured with SRBC, the frequency of specific antigen-binding cells (ABC) either did not change or, at most, increased threefold. The addition of LPS or aggregated IgG to the cultures did not alter this, although the numbers of antibody-secreting cells recovered from the cultures were markedly increased by LPS and decreased by IgG. In contrast, when fetal liver cells were cultured with SRBC and LPS or aggregated IgG, the numbers of SRBC-ABC formed exceeded that found in cultures of adult cells, but there was no change in the small number of PFC produced.

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Mechanisms of Glucocorticoid-Induced Osteopenia: Role of Parathyroid Glands[1] (39396)

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Excessive glucocorticoids can cause osteopenia in animals (1) and man (2). However, the mechanisms by which they produce this effect are not entirely clear. *In vitro* studies have shown that glucocorticoids inhibit vitamin A or parathyroid hormone (PTH)-induced bone resorption (3). *In vivo* studies have shown that glucocorticoids inhibit bone formation (4, 5). However, the bone resorption studies *in vivo* have given conflicting results (5, 6). In studies which showed increased bone resorption due to glucocorticoids, excessive PTH has been postulated as one of the mechanisms of this increase. Excessive PTH secretion has been shown to occur in rats (7) and in man (8) after acute or chronic cortisol administration.

Recent studies have indicated that the glucocorticoids' effect on bone resorption may be dosè-dependent, low doses causing increased resorption and high doses causing decreased or normal resorption (9, 10). The increased bone resorption was again postulated to be the result of excessive PTH secretion.

The present studies were conducted to evaluate the role of parathyroid glands in the production of glucocorticoid-induced osteopenia.

Materials and Methods. Sprague-Dawley rats initially weighing 260-290 g were maintained on a diet containing 1.7% calcium and 1.5% phosphorus. All rats were injected with 45 μCi of ^{45}Ca ip in three divided doses at 3-day intervals. Ten days after the last dose of ^{45}Ca, half the rats were parathyroidectomized (PTX) and the other half were left intact. Parathyroidectomy was confirmed by serum calcium levels of less than 7.0 mg/100 ml and undetectable serum PTH levels.

The animals were divided into four groups 1 week after the parathyroidectomy, at which time injections were begun and continued daily for 17 weeks. Group I: Intact rats were given normal saline sc daily to serve as control ($n = 6$). Group II: Intact rats were given cortisol (Cortef, Upjohn Company, Kalamazoo, Mich.), 5 mg/kg/day sc ($n = 6$). Group III: PTX rats were given normal saline sc daily ($n = 5$). Group IV: PTX rats were given cortisol, 5 mg/kg/day sc ($n = 4$).

The rats were kept in individual metabolic cages. The animals were bled by orbital sinus puncture at 2 weeks, 12 weeks, and at the end of the study at 17 weeks. The serum was separated and analyzed immediately for ^{45}Ca activity and frozen for subsequent determinations of calcium, phosphorus, and PTH.

Two weeks after beginning the injections, the feces were collected for two successive 72-h periods and then ashed. The ash was dissolved in hydrochloric acid for analysis of ^{45}Ca activity.

At the end of 17 weeks the rats were killed by ether anesthesia and the right femur was removed. The bone was quickly freed of soft tissue and weighed. The length was determined by verneir calipers. The bones were then bisected with a fine saw and freed of the marrow tissue with a jet of water. The bones were dried in crucibles overnight in an oven at 100°, and the dry weight obtained. The bones were then ashed in a muffle furnace at 800° overnight and the ash weight was obtained. The bone ash was dissolved in hydrochloric acid for deter-

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[1] Note: This work was presented in part at the Annual Meeting of the Midwestern Section of the American Federation for Clinical Research on October 31, 1974, Chicago, Illinois.

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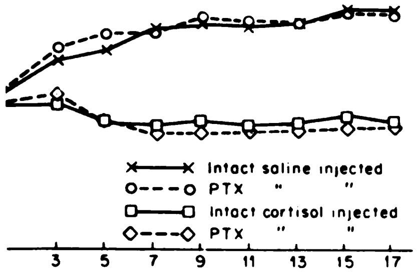
ɔn of calcium content and ^{45}Ca activ-

calcium and phosphorus determina- were made by the autoanalyzer ꞁ of Kessler and Wolfman (11). The :tivity determinations were made by ing the samples in Bray's scintillation n (12) and counting them in a Pack- ;uid scintillation counter. Sufficient were accumulated to reduce the ıg error to 2% or less in all samples. PTH determinations were made by lioimmunoassay method of Hargis *et*). The data were analyzed by Stu- *t* test. The statement of significant ıce denotes a *P* value of less than

lts. There were no group differences ıitial body weights of the rats. Saline- ꞁ PTX and intact rats progressively weight and had similar final weights). The cortisol-treated intact and ıts lost minimal weight initially and mained stable between 90 to 95% of ial body weight (Fig. 1).

·e 2 shows the serum PTH values of ıct rats. The cortisol-injected group nificantly greater serum PTH levels ıree time periods studied.

n calcium and phosphorus determi- made at the time of bleeding at 17 ıre shown in Table I. There was no ınt difference between the serum of the saline-treated and cortisol- intact animals. Cortisol tended to e the serum calcium in the PTX rats h the decrease did not reach statistical significance. As expected, the serum phosphorus was significantly greater in the PTX animals than in the intact animals.



. Effect of cortisol injections or of normal :ctions on the body weight of intact and PTX results are expressed as a percentage of the ly weight. Each point represents the mean r the group at each time shown on the ab-

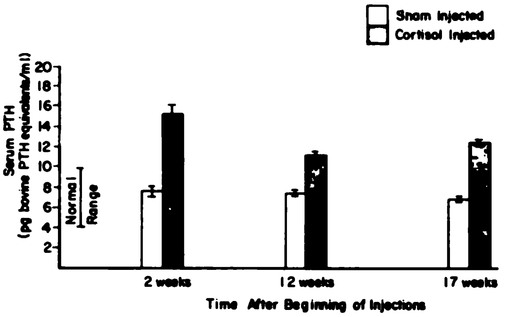


FIG. 2. Effect of cortisol injections or of normal saline injections on the serum PTH concentration in intact rats at 2, 12, and 17 weeks after the beginning of the injections. Each bar represents the mean ± SE for the group.

The results of serum ^{45}Ca expressed as total counts per minute per milliliter of serum are shown in Fig. 3. Cortisol treatment significantly reduced the serum ^{45}Ca activity in both the intact and the PTX rats except at 17 weeks in the intact group.

The fecal ^{45}Ca activity expressed as counts per minute per 24 hours is shown in Fig. 4. The PTX saline-injected rats had significantly less fecal ^{45}Ca as compared to intact saline-injected rats. Cortisol treatment significantly decreased the radioactivity in the feces of both intact and PTX rats.

The data pertaining to bone are portrayed in Table II. The PTX saline-injected rats had higher ash weight and percentage ash content (ash weight × 100)/(dry weight) as compared to intact saline-treated rats. Cortisol treatment reduced the percentage ash content in both intact and PTX rats (Table II). The ash weight of bone when expressed as per centimeter length of bone (ash weight)/(length in cm) was significantly greater in PTX saline-injected rats as compared to intact saline-injected rats and was reduced significantly by cortisol treatment in both intact and PTX rats.

The total ^{45}Ca activity in the bone was significantly greater in PTX saline-injected rats as compared to intact saline-injected rats. An effect of cortisol treatment on total bone ^{45}Ca could not be detected in either the intact or PTX groups despite the decrease in the serum and fecal ^{45}Ca (presumably because the great amount of radioactiv-

TABLE I. EFFECT OF CORTISOL INJECTIONS OR OF NORMAL SALINE INJECTIONS ON SERUM CALCIUM AND PHOSPHORUS AT 17 WEEKS AFTER BEGINNING OF INJECTIONS IN INTACT AND PTX RATS.[a]

Group	Serum calcium (mg/100 ml)	Serum phosphorus (mg/100 ml)
I. Intact saline injected	9.66 ± 0.05	4.9 ± 0.31
II. Intact cortisol injected	9.67 ± 0.14	4.71 ± 0.31
III. PTX saline injected	7.10 ± 0.20	8.76 ± 0.52
IV. PTX cortisol injected	6.67 ± 0.20	9.96 ± 0.84

[a] All values are expressed as the mean ± SE.

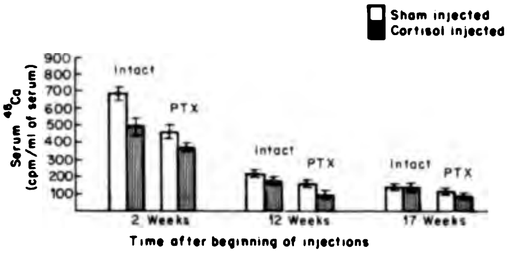


FIG. 3. Effect of cortisol injections or of normal saline injections on serum ^{45}Ca in intact and PTX rats at 2, 12, and 17 weeks after the beginning of the injections. Each bar represents the mean ± SE for the group.

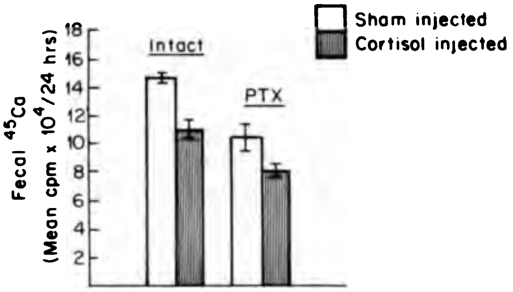


FIG. 4. Effect of cortisol injections or of normal saline injections on the fecal ^{45}Ca in intact and PTX rats at 2 weeks after the beginning of the injections. Each bar represents the mean ± SE.

ity in bone precluded detecting smaller changes secondary to decrease in fecal ^{45}Ca). Bone specific activity (^{45}Ca cpm/mg of Ca) was significantly increased by cortisol treatment in PTX rats. The intact rats showed a similar trend, although the results were not statistically significant.

Discussion. The observation of increased serum PTH induced by cortisol in the present study is in agreement with the previous studies in rats (7) and in man (8). Glucocorticoids decrease the intestinal absorption (14) and increase the renal excretion of calcium (15), and therefore can secondarily increase PTH secretion. The other mechanism by which serum PTH could be increased by cortisol is by inhibition of bone resorption with a tendency toward hypocalcemia causing compensatory increase in PTH. *In vitro* studies have shown that cortisol inhibits vitamin A or PTH-induced bone resorption (3). In this study a decrease in serum and fecal ^{45}Ca and an increase in bone ^{45}Ca specific activity were interpreted as evidence of decreased bone resorption *in vivo*. By these criteria, bone resorption was inhibited by cortisol treatment in the PTX rats. In the intact animals injected with cortisol, decreased bone resorption was suggested by decreased serum and fecal ^{45}Ca activity at 2 weeks and decreased serum ^{45}Ca activity at 12 weeks. However, the serum ^{45}Ca activity was not decreased by cortisol at 17 weeks, and the bone ^{45}Ca specific activity was not significantly increased in these intact animals. These observations suggest that the effect of cortisol to decrease bone resorption had been overcome by the compensatory increase in PTH secretion.

In the present study the bone mass was determined by dry and ash weights of the bone. It was significantly increased in the PTX saline-injected rats as compared to intact saline-injected rats. This was probably on the basis of decreased bone resorption in the former group, in the absence of PTH. Cortisol-treated animals were significantly smaller in size as compared to their respective saline-injected controls, and therefore the ash and dry weights of the bones cannot be directly compared. To obviate this, the dry and ash weights were compared on the basis of per centimeter length of the bone. Dry and ash weights expressed in this manner were significantly reduced by cortisol treatment in both the intact and the PTX rats as compared to their respective controls. Percentage content has been shown to decrease in osteoporosis (16). In the present study cortisol treatment significantly re-

E II. EFFECT OF CORTISOL INJECTIONS OR OF NORMAL SALINE INJECTIONS ON VARIOUS PARAMETERS OF THE RIGHT FEMUR IN INTACT AND PTX RATS.[a]

·oup	Dry weight (mg)	Ash weight (mg)	Ash (%) [(Ash weight/ Dry weight) × 100]	Dry wt in mg/Length in cm	Ash wt in mg/Length in cm	Total calcium (mg)	Total ^{45}Ca (cpm × 10^{3})	Specific activity (Total ^{45}Ca cpm/Calcium in mg)
aline injected	658.8	436.7	66.29	169.8	113.2	162.2	1017.5	6280
	± 10.7	± 6.1	± 0.32	± 2.1	± 1.2	± 2.3	± 12.4	± 127
ortisol injected	579.8	371.3	64.02	159.7	102.3	137.8	940.4	6820
	± 10.5*	± 8.9*	± 0.53*	± 2.0*	± 1.9*	± 3.8*	± 61.4	± 411
line injected	712.7	495.7	69.54	183.5	127.6	181.6	1105.9	6131
	± 14.3**	± 10.6**	± 0.20**	± 3.0**	± 2.3**	± 6.8**	± 29.7**	± 326
rtisol injected	597.8	391.3	65.43	167.5	109.7	145.5	1091.1	7480
	±'8.3***	± 8.8***	± 0.56***	± 2.3***	± 2.4***	± 2.6***	± 64.7	± 321***

ıes are expressed as the mean ± SE.
s that Group II values are significantly different ($P < 0.05$) from Group I.
es that Group III values are significantly different ($P < 0.05$) from Group I.
ıtes that Group IV values are significantly different ($P < 0.05$) from Group III.

:he percentage ash content in both
nd PTX rats.

dicated above, cortisol injections re-
he bone resorption in PTX rats and
a tendency toward decreased resorp-
the intact rats. In spite of this, the
ıass was reduced by cortisol injec-
ı both these groups. These studies
herefore indicate that in rats (i) cor-
mg/kg/day, induces osteopenia by in-
of bone formation, and (ii) the pres-
parathyroid glands is not essential
ıduction of cortisol-induced osteo-

ıbservations in the present study are
ible with the hypothesis by Jee (9)
ımussen (10) that high doses of corti-
5 mg/kg/day, or greater, induce os-
a predominantly by inhibition of
rmation.

nary. Intact and PTX rats previously
l with ^{45}Ca received cortisol, 5 mg/
for 17 weeks. Bone resorption as
ned by serum and fecal ^{45}Ca and
Ca specific activity were reduced by
in the PTX rats and showed a simi-
lency in intact rats. In spite of this,
ıe mass, as determined by ash con-
as reduced by cortisol in both the
nd PTX animals. The data show that
isol, 5 mg/kg/day, produces osteo-
y inhibition of bone formation, and
presence of parathyroid glands is not
l for the production of this osteo-

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Age-Related Alteration in Human Heart Collagen[1] (39397)

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Hamlin and Kohn (1) have reported a decreased rate of collagenase digestion of insoluble human tendon collagen with increasing age. Tendon was used as an abundant source of collagen that can be readily purified.

Aging in mammals is accompanied by a decline in efficiency of virtually all physiological processes (2–4). A hypothesis has been proposed (5) explaining the debilities of aging using the collagen cross-linking model. This hypothesis suggests that insoluble collagen found in parenchymatous tissue should exhibit age-specific alterations similar to that found in human tendon.

We now report on the presence of such age-specific alterations in insoluble human myocardial collagen.

Methods and materials. Heart tissue source. Hearts were obtained at autopsy from male accident victims. The hearts were selected from two age groups: (i) 10 hearts 18- to 28-yr-old (mature group); and (ii) 6 hearts 62- to 91-yr-old (old group). Hearts directly involved in disease processes were not used for study. Hypertrophied hearts and hearts showing gross evidence of infarcts and/or fibrosis were excluded. Heart samples were obtained 2–6 hr after death and were stored at −40° until processed. Studies with fresh tendon and with purified collagen showed that storage did not significantly affect digestibility. There were no differences in storage and handling of samples in the two age groups.

Heart collagen purification. All procedures were performed at 5° unless stated otherwise. The left ventricle, including the interventricular septum, was excised. The epicardium and endocardium were dissected away, along with any visible fat, valves, blood vessels, and connective tissue. Fifty grams (wet weight) of myocardium were then pressed under a 40,000-lb ram force in a hydraulic press (Model SP-215, Pasadena Hydraulics, Inc., El Monte, California). The pressed tissue was placed immediately in 500 ml of 0.15 M NaCl in 0.01 M Tris-HCl (pH 7.4) and shaken vigorously on an agitator until repeated washings with the NaCl–Tris solution became clear. This usually occurred after 5 days with NaCl–Tris changes every 12 hr. The residue was then similarly washed with 1.0 M NaCl in 0.01 M Tris–HCl (pH 7.4). A few drops of toluene were added to the NaCl–Tris stock solutions to prevent bacterial growth. At the time of each wash change, the suspension was passed through a No. 60 U.S. Standard Testing Sieve (The W.S. Tyler Co., Cleveland, Ohio) with an opening of 250 μm. The material remaining on the sieve was removed and macerated for 2 min with a mortar and pestle and was then resuspended in 500 ml of the appropriate NaCl-Tris solution. Finally, this material was washed twice in 500 ml of distilled water with sieving after each wash. The material remaining on the sieve was removed and frozen at −40° until further processing.

The fraction passing through the sieve was pooled for each heart sample processed. These pooled washes were centrifuged at 25,000–30,000 g for 120 min. The resulting supernatants were discarded and the precipitates were frozen at −40°, lyophilized, and stored at −40°. This fraction was subsequently analyzed for collagen so that an estimate of total myocardial collagen content as well as percentage collagen recovered could be obtained.

The material frozen after isolation on the sieve was lyophilized and treated as previously described (1, 6), with the following modifications: Lipid was extracted in three changes of petroleum ether for 1, 3, and 24 hr, respectively. A total of four washes with 1.0 M NaCl in 0.01 M Tris–HCl (pH 7.4) and six final washes with distilled water were performed. The material remaining

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these procedures was frozen at −40° yophilized. This material constituted urified human heart collagen (purified ial).

lagen content of purified material. aliquots of 20 mg of purified mate- omogenized in 1 ml of distilled water a ground-glass homogenizer), were ted twice with 5 ml of 5% TCA at 90° min. The extracts were centrifuged at 0 *g* for 15 min. The fraction isolated he pooled washes passing through the was treated similarly. Hydroxyproline it in aliquots of supernatant were de- ıed by the method of Stegeman and r (7). Collagen content was deter- assuming 14% hydroxyproline by t.

agen digestion. Clostridial collagenase btained from the Worthington Bio- cal Corp. (Lot CLSPA 2KA, 440 U/ nd prepared as previously described he collagen digestions, with purified al containing 10 mg of collagen, were red in a pH-stat as reported previ- (1, 6), with the following modifica- The reaction volume was 15 ml and ed of 10 mg of collagen, 400 units of nase, 2 ml of distilled water, and 13 0.1 *M* salt solution (0.05 *M* in $CaCl_2$ .05 *M* in NaCl). The reaction pro- l for 45 min, at which time it was d by freezing in an acetone–Dry Ice t −70°. The reaction mixtures were tored at −40° until further analysis. eterminations were thus of rate of on, or of amount digested per 45 min. ion curves are not linear over this eriod (1), but the amount digested is a function of age (6). Control incuba- ere done on aliquots of three samples ified collagen from both mature and ups, excluding the collagenase. Also, tions were done on three aliquots of nase, excluding the collagen. The ls were otherwise treated in the same r as the experimental group.

stion product analysis. The reaction es were thawed and centrifuged at 0 *g* for 60 min at 5°. The digestion ts were passed through an ultrafiltra- embrane (Amicon PM 10; Lexington, , using an ultrafiltration cell (Amicon 12) to exclude collagenase. Aliquots of the digestion product filtrates were hydrolyzed in 6 *N* HCl at 110° for 18 hr. The hydrolyzed samples were then analyzed for hydroxyproline content as above and for ninhydrin reactive material by the method of Rosen (8). L-Leucine was used as the standard for the ninhydrin determinations.

Collagen standards. Three aliquots of 23-yr-old (>95% collagen) and 88-yr-old (>95% collagen) purified human diaphragm-tendon collagen were hydrolyzed as above and analyzed for hydroxyproline content and ninhydrin-reactive material. Three aliquots of hot TCA-extracted 18-yr-old (31.1% collagen) and 91-yr-old (41.4% collagen) purified human heart collagen were treated similarly. A ratio of total micrograms per milliliter of hydroxyproline to total micrograms per milliliter of ninhydrin-reactive material was determined. This served as a standard against which to measure the digestion products for evidence that the digestion products were a result only of enzymatic hydrolysis of collagen.

Statistical analysis. The differences between the means were compared using the Student's *t* test.

Results. The collagen concentration of mature hearts is greater than old hearts on a wet weight basis (Table I). No age-specific differences exist in the percentage of collagen recovered and in the percentage of collagen present in purified material (Table I).

The digestion rates of mature collagen are much greater than that for old collagen (Table I). Additionally, the amounts of hydroxyproline and ninhydrin-reactive material, per milliliter of digestion product filtrate, are greater in mature than in old samples. Filtrates from the incubated collagen and collagenase controls contain ninhydrin-reactive material. Recalculating hydroxyproline to ninhydrin ratios, after subtracting control ninhydrin values from experimental values, gives ratios of 0.159 and 0.167 for filtrates from mature and old groups, respectively. Thus, on the basis of hydroxyproline content, the digestion products represent collagen at least as pure as that from tendon (Table II).

Digestion in collagen controls is minimal, does not involve hydroxyproline-containing protein, and does not show an age-difference (Table I). Hydroxyproline to ninhy-

TABLE I. Determinations in Mature and Old Myocardial Samples.

	Experimentals		Controls[a]		
	Mature	Old	Mature collagen	Old collagen	Collagenase
Sample number[b]:	10	6	3	3	3
Collagen content of myocardium (% wet wt)	0.63 ± 0.03[d]	0.44 ± 0.05	–	–	–
Total collagen recovered (% dry wt)	85.8 ± 1.2	82.0 ± 1.4	–	–	–
Collagen content of purified material (% dry wt)	39.1 ± 2.1	39.4 ± 2.6	–	–	–
Digestion (μl NaOH)[c]	737 ± 9[d]	549 ± 26	8.0 ± 1.3	9.5 ± 0.3	18 ± 1.6
Hydroxyproline content of filtrates (μg/ml)	70.3 ± 0.8[d]	54.5 ± 2.2	0	0	0
Ninhydrin-reactive material content of filtrates (μg/ml)	584.1 ± 14.9[d]	447.5 ± 17.2	74.5 ± 14.1	53.2 ± 6.2	67.3 ± 7.4
Hydroxyproline to ninhydrin ratios	0.121 ± 0.004	0.122 ± 0.002	0	0	0

[a] Collagen controls consist of 10 mg of collagen from purified material which was placed in the reaction vessel under identical conditions as the experimentals except for excluding collagenase. Collagenase controls consist of 400 units of collagenase in the reaction vessel under identical conditions as the experimentals except for excluding collagen.

[b] Three aliquots of each sample were determined and an average value of all aliquots was obtained. All values are mean ± SE.

[c] Denotes the amount of 0.01 *M* NaOH added to reaction mixture during 45-min incubation.

[d] Value significantly different ($P < 0.01$) from corresponding old experimental value.

drin-reactive material ratios are very similar in mature and old highly purified human tendon collagen, the digestion products from purified material of mature and old human myocardium, and collagen extracted by TCA from mature and old heart (Table II).

Discussion. Noncollagenous material is present in the digestion product filtrates, as evidenced by the ninhydrin values of the collagen and collagenase controls. However, comparing ratios of hydroxyproline to ninhydrin reactive material in the digestion product filtrates and in highly purified human tendon collagen indicates the contribution of noncollagenous material is trivial, and that virtually all of the digestion is the result of collagen degradation and not of nonspecific proteolytic activity in the two age groups.

The data demonstrate the presence of age-specific alterations in insoluble collagen of human myocardium. The digestion rates and changes with age are similar to those found in human tendon, as previously reported (1). This suggests that increasing stabilization of collagen with age may be generalized throughout the body. Although the nature of the molecular alterations in aging collagen is not known, it has been suggested that they consist of intermolecular cross-linking, and it is assumed that such alterations are manifested by the decreased digestion rates with collagenase.

TABLE II. Ratios of Hydroxyproline to Ninhydrin-Reactive Material from Different Collagen Sources.

Collagen source[a]	Ratio[b]
Mature human tendon	0.126 (0.122 to 0.129)
Old human tendon	0.126 (0.120 to 0.134)
TCA extract of mature purified material[c]	0.123 (0.120 to 0.125)
TCA extract of old purified material	0.129 (0.128 to 0.129)
Mature digestion product filtrate[d]	0.121 (0.105 to 0.128)
Old digestion product filtrate	0.122 (0.111 to 0.127)

[a] For each mature and old collagen source, three aliquots of one sample were determined.

[b] Values are means of aliquot values with the range given in parentheses.

[c] Purified material from human myocardium, containing 10 mg of collagen, was extracted with hot TCA. The TCA extract was analyzed for hydroxyproline and ninhydrin-reactive material.

[d] Digestion product filtrate from purified material of human myocardium.

Rat heart becomes less compliant with increasing age (9). It was suggested that connective tissue might be responsible for this change. Kohn and Rollerson (10) obtained data suggesting that the increased rigidity of human myocardium was due to increased rigidity of intercellular collagen. Osmotic swelling ability was used as a measure of this rigidity. It was postulated that this increased rigidity may be due to progressive formation of intermolecular bonds between collagen molecules.

Myocardial rigidity resulting from alterations in collagen could result in decreased myocardial contractility which may explain, in part, the physiologic decline in cardiac function known to occur with age (2).

Additional data are required to determine if sites of enzymatic cleavage are similar in the two age groups studied. Analysis of the unhydrolyzed digestion product filtrates would be useful in this regard.

Summary. A technique for the purification of insoluble human myocardial collagen is described. The digestion rate of old collagen with collagenase is much less than that of mature collagen. These findings are similar to those in human tendon collagen. It is suggested that progressive stabilization of collagen with age may be a generalized phenomenon, and responsible, in part, for the decline of myocardial function with age.

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A Simple Method for Preparing Suspensions of Luteal Cells[1] (39398)

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Methods for preparing suspensions of isolated cells have been developed for a number of tissues, including the adrenal (1–4), the anterior pituitary (5–11), the ovary (4, 12, 13), the pancreas (14), the testis (15), and the thyroid (16). The use of cell suspensions for biological studies offers several advantages including: (i) the physical and hormonal environment of the cell can be easily controlled and monitored; (ii) many biochemical parameters which can not be measured *in vivo* are more easily approached *in vitro;* and (iii) the ability to distribute cell suspensions homogeneously alleviates much of the variability associated with other *in vitro* systems. A variety of enzymatic procedures has been used to disrupt the tissue matrix and free the cells. The only method described for luteal tissue (12) employed a number of enzymes in a rather complex procedure. The following communication describes a simple and highly reproducible procedure for the dissociation of ovine corpora lutea into isolated cell suspensions.

Materials and Methods. Cells dissociation. Corpora lutea from ewes, superovulated as described by Sheridan *et al.* (17), were collected and placed immediately into iced Medium 199 (Grand Island Biological Company) containing 25 m*M* *N*-2-hydroxyethylpeperazine-*N*′-2-ethanesulfonic acid (HEPES, Sigma Chemical Company). Adherent connective tissue was removed from the corpus luteum and 0.5-mm slices were prepared with a Stadie-Riggs tissue slicer. The first and last slice of each corpus luteum was discarded to minimize capsular material. The slices were weighed and transferred to a spinner flask, and Medium 199 was added (5 ml/g tissue). The flask was placed in a water bath (32°), and the contents were stirred magnetically (100 rpm) for 10 min. The medium was then decanted, fresh medium with collagenase (2000 U/g tissue, Type I CLS, Worthington Biochemical Corporation) was added, and the incubation continued. At frequent intervals the medium was examined microscopically to evaluate the progress of the dissociation. When a few free, viable cells began to appear (30–40 min), the heavier material was allowed to settle and the supernatant was decanted and discarded. Fresh medium with enzyme was added and the dissociation was continued. When the slices were reduced to small clumps, the dissociation was hastened by repeatedly drawing these clumps into a pasteur pipet. The procedure was stopped when free cells predominated in the medium (2 hr). The contents of the flask were transferred and centrifuged (100 *g*, 5 min), the supernatant was discarded, and the cells were washed six times with fresh medium. The cells were resuspended and filtered through four layers of cheese cloth to eliminate large particles. This final preparation was evaluated for cell concentration and viability. If the suspension was not used immediately, it was stored at 4° with added antibiotics (100 U of penicillin G and 100 μg of streptomycin sulfate/ml, Difco Laboratories).

Viability of cells was estimated with a dye exclusion test using 0.2% trypan blue in 0.9% saline. Cells excluding the dye were considered viable (18). Cells were routinely counted in a standard hemocytometer and

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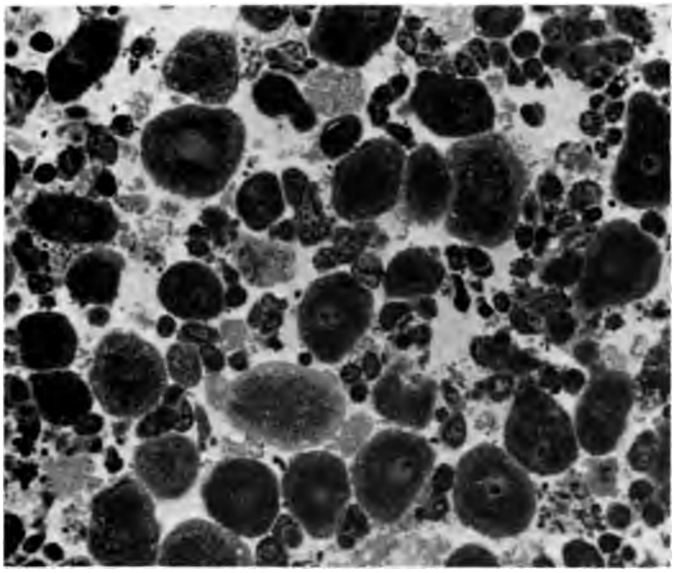
ied as small (av diam 9 μm) or large
iam 28 μm). Each preparation was
ted to a standard concentration. Ap-
nately 30–40 × 10^3 viable large cells
l were used to study progesterone syn-
.

gesterone synthesis. Preparations of
× 10^3 viable large cells per ml (1–1.2
total cells) were dispensed in 0.4-ml
ts to iced, round-bottom culture tubes
85 mm). NIH-LH-S16, NIH-prolac-
, and NIH-HCG-CR117 were added
μl of phosphate (0.05 *M*) buffered
(0.14 *M*) with 0.1% gelatin at pH 7.0
gel). The tubes were incubated at 37°
Dubnoff Metabolic Shaking Incubator
1 hr. The incubations were terminated
nsferring the tubes to an 80° water
or 20 min to kill the cells and denature
teroid enzyme systems. The samples
liluted with 8.6 ml of PBS-gel. Proges-
concentrations were determined by
mmunoassay (19). In a preliminary
ment, 15 samples were extracted with
listilled chloroform and the progester-
the extract was purified on silica gel-
nated glass fiber sheets (Gelman In-
s, Inc.) using 10 cyclohexane:1 ethyl acetate. Progesterone levels in aliquots of the media were identical to those obtained after extraction and chromatographic purification of the samples. Therefore, the incubation media were assayed directly for subsequent studies.

Results. The first cells to appear free in the medium, other than erythrocytes, were small cells, possibly fibroblasts. These cells predominate in the final cell suspension and range from 5 to 13 μm (av 9 μm) in diameter. The first large cells to appear were not viable, but the proportion of free, viable, large cells gradually increased as the tissue was dissociated. The large cells ranged from 22 to 37 μm (av 28 μm) in diameter. The average yield of total cells from tissue obtained 11 days after superovulation (17) was 81.8 ± 9.1 × 10^6 cells/g tissue. There were 3.9 ± 0.3 × 10^6 large cells/g tissue. The micrograph in Fig. 1 shows a representative preparation after fixation and staining. The trypan blue dye exclusion test indicated that 75–85% of all cells were viable. Acridine orange gave comparable results.

The dissociated cells contained substantial quantities of progesterone at the beginning of the incubation. The addition of lu-



1. Dissociated cells from corpora lutea of a superovulated ewe on Day 9 of the estrous cycle. The cells
ed in a pellet with 4% glutaraldehyde, postfixed in osmium and embedded in epon. A 1-μm section was
stained with toluidine blue. (× 750).

teinizing hormone (LH) or human chorionic gonadotropin (HCG) to the cells stimulated a dramatic increase in progesterone output; however, the cells did not respond steroidogenically to prolactin (Table I). Increasing doses of LH produced a differential steroidogenic response which was maximal around 10 ng per incubated tube (Table II).

The initial progesterone content, the progesterone production with time, and steroidogenic response to LH all varied proportionately with cell number (Table III).

To determine if dissociated cells could be stored, paired samples of cells were incubated immediately or stored at 4° for 48 hr in Medium 199 or in 50% Medium 199 and 50% hypophysectomized sheep serum (LH free). The stored cells were washed and incubated as described above. Both groups of stored cells produced less progesterone than the fresh preparation and their steroidogenic response to LH was reduced although the percentage increase above unstimulated controls was similar to that of the fresh preparation (Table IV).

Superovulated animals frequently had luteinized follicles and the cells from these follicles were dissociated to determine their steroidogenic capacity. Luteal tissue from the same animal was dissociated and incubated concurrently. There was no substantial difference in progesterone production (Table V) by these two cell preparations.

Luteal tissue from other species has been dissociated and the technique works well with bovine luteal tissue. However, the procedure is too harsh for use with rabbit or rat luteal tissue. It requires modification with respect to enzyme concentration and dissociation time before it can be used efficiently with tissue from these species.

Discussion. A variety of enzymes has been utilized to obtain suspensions of cells from endocrine tissues. The most extensive research regarding the dissociation of luteal tissue is that of Gospodarowicz and Gospodarowicz (12, 20) and Papaionanou and Gospodarowicz (21), who used a complex combination of enzymes and produced a pure population of large luteal cells. However, the population of cells produced is not representative of the cells which comprise the corpus luteum *in vivo*. This must have resulted from procedural damage to certain cell types which did not appear in the final preparation. The cells which remained may have also suffered enzymatic damage. Their cell preparations were responsive to LH simulation and appear to provide an excellent product for studying the interactions of gonadotropins and the large luteal cells.

The advantage of the procedure we have described is its simplicity and the fact that the population of cells obtained is typical of that found *in vivo*. Fifty-two percent of the volume of the ovine corpus luteum, during the midluteal phase of the cycle, is occupied by luteal cells (35% granulosa-lutein, 17%

TABLE I. PROGESTERONE PRODUCTION BY LUTEAL CELLS AND THE EFFECT OF GONADOTROPINS ON PROGESTERONE PRODUCTION.

Treatment	Incubation (hr)	Progesterone production (ng)
None	0	237 ± 20
None	1	281 ± 17
LH (100 ng)	1	627 ± 37^{a}
Prolactin (1 μg)	1	291 ± 11
None	0	105 ± 34
None	1	124 ± 10
HCG (25 ng)	1	231 ± 52^{a}

Note: Values = Mean ± SD.

[a] Significantly different from respective 1-h control, $P < 0.01$.

TABLE II. PROGESTERONE PRODUCTION IN RESPONSE TO VARIOUS LEVELS OF LH.

Treatment (ng)	Incubation (hr)	Progesterone production (ng)	
		Expt. 1	Expt. 2
None	0	96 ± 25^{a}	75 ± 7^{a}
LH (0.1)	1	117 ± 8^{ab}	110 ± 11^{b}
LH (0.3)	1	126 ± 6^{ab}	153 ± 8^{c}
LH (1.0)	1	161 ± 13^{ab}	197 ± 9^{d}
LH (3.0)	1	204 ± 11^{cd}	194 ± 7^{d}
LH (10.0)	1	254 ± 9^{d}	250 ± 25^{e}
LH (30.0)	1	180 ± 46^{c}	262 ± 25^{eg}
LH (100.0)	1	188 ± 31^{c}	297 ± 4^{f}
LH (300.0)	1	207 ± 25^{cd}	287 ± 44^{fg}

Note: Means with different superscripts are significantly different ($P < 0.05$). Values = Mean ± SD.

TABLE III. EFFECTS OF CELL CONCENTRATION ON PROGESTERONE SYNTHESIS.

		Progesterone production (ng)		
nent	Incubation (hr)	60 K/ml	24 K/ml	12 K/ml
	0	130 ± 12^a	41 ± 4^b	18 ± 3^b
	1	175 ± 23^c	75 ± 5^d	31 ± 5^b
μg)	1	656 ± 74^e	261 ± 3^f	83 ± 6^g

ans with different superscripts are significantly different ($P < 0.05$).

TABLE IV. EFFECTS OF CELL STORAGE ON PROGESTERONE PRODUCTION.

		Progesterone production (ng)		
			After storage[2]	
ıent	Incubation (hr)	Original[1]	WHSS[3]	Medium 199[4]
	0	110 ± 22^a	26 ± 2^d	90 ± 14^a
	1	228 ± 32^b	102 ± 26^a	121 ± 8^a
μg)	1	502 ± 3^c	371 ± 51^e	341 ± 35^e

ues = Mean ± SD. Means with different superscripts are significantly different ($P < 0.05$).
pension used immediately after dissociation.
for 48 hr at 4°.
was ½ Medium 199 and ½ hypophysectomized sheep serum.
edium 199.

/. PROGESTERONE PRODUCTION BY CELLS
EINIZED FOLLICLES OR CORPORA LUTEA.

Incubation (hr)	Progesterone production (ng)	
	Luteinized follicles	Corpora lutea
0	82 ± 7^a	126 ± 8^a
1	155 ± 9^b	202 ± 19^b
1	656 ± 31^c	558 ± 56^d

lues = Mean ± SD. Means with different
are significantly different ($P < 0.05$).

in). The remainder of the volume
ive tissue (35%) and blood vascu-
(13%) (22). Our cell preparations
.8% large cells (av diam 28 μm)
% small cells (av diam 9 μm).
these figures, the large luteal cells
parations represent 59.7% of the
olume which approximates the *in*
ition. Under experimental condi-
ere interaction between different
or their secretory products may
the function of a particular popu-
ells, it is important that the disso-
s be representative of the normal

ociate the ovine corpus luteum,
zymes were tried, including crude
urified trypsin, subtilisin, hyaluronidase, crude collagenase, and purified collagenase. The best results were obtained with crude collagenase which was twice as effective as its highly purified form with respect to dissociation time. This indicates that other proteolytic enzymes in the preparation were beneficial. Various concentrations of enzymes were tested, but the most extensive trials were made with crude collagenase (300–4000 U per g of tissue). The addition of fresh collagenase at frequent intervals did not alter dissociation time or cell yield.

Krebs ringer, bicarbonate buffer plus glucose (KRBG) with one-half the normal Ca^{2+} failed to maintain the cells through the dissociation and incubation procedures. Fortification of KRBG with 4% bovine serum albumin (BSA) (12) produced satisfactory results; however, there are disadvantages to the use of BSA. BSA contains a small, but variable, amount of LH which would confound any studies of progesterone production. Four lots of commercial BSA were assayed for LH by radioimmunoassay and found to contain <2, <2, 8.3, and 14.5 pg/mg of BSA. BSA and other common cell culture additives, such as fetal calf serum (FCS), also protect the cells from stains such as trypan blue and result in erroneously high viability ratings. Using BSA or FCS, the estimated viability of cells was 95%, whereas without BSA or FCS, viabilities were reduced to the normal range. When cell preparations with viabilities as low as 65% were resuspended in medium containing BSA or FCS, they had viability ratings of 90-95%. Tennant (18) showed that serum protected cells against the toxic effects of trypan blue and that cells rendered metabolically inert by heat treatment produced excellent viability ratings in the presence of serum. For these reasons, defined media such as Dulbecco's Modified Eagles and Medium 199 were selected.

Cell suspensions, prepared as described, synthesize progesterone and are stimulated to increase progesterone output in response to either LH or HCG, indicating the presence of functional luteal cells. Cell preparations vary with respect to steroidogenic capabilities, possibly due to animal-to-animal variation. Sayers *et al.* (14) felt that similar variability in adrenal cell suspensions may have resulted from heterogeneous cell populations. Total cell counts would fail to reflect these differences; however, in the present study cell counts were standardized on the basis of large luteal cells.

Increasing doses of LH elicited a graded response in progesterone production. This system could serve as a bioassay for LH or HCG. The concentration of LH (10^{-8} *M*) at which maximum progesterone production was reached compares favorably with that found by Gospodarowicz (12) (10^{-7} *M*) for maximum progesterone synthesis and Kammerman (23) (10^{-8} *M*) for maximum binding of HCG. When the cell concentration was varied, the progesterone synthesis and the response to LH varied proportionately, indicating that the medium was adequately supplying the requirements of the cells during incubation. Cells stored at 4° for 48 hr after dissociation maintained viability and responded steroidogenically. The steroidogenic response, however, was not so great after 48 hr as when the preparation was fresh. This decrement could signify cell deterioration or a gradual loss of differentiated function as a result of culturing techniques.

Procedures for separating dissociated luteal cells into the cell populations described in this report are presently being developed. Centrifugation on both isopycnic and isokinetic gradients composed of sucrose, Ficol, or Metrizamide have been employed with limited success. The most promising method appears to be the separation of cell types on a Sepharose 2B-300 column. Using this procedure, it has been possible to obtain functional small lutein cells devoid of contamination by large cells.

Summary. A simple and highly reproducible method was developed for the dissociation of ovine luteal tissue into isolated cell suspensions using a crude collagenase preparation in Medium 199. The procedure required less than 3 hr. The mean yield of cells was 81.8 ± 9.1 × 10^6 cells/g tissue. The resulting cell preparation synthesized progesterone and responded to the addition of LH or HCG, but not prolactin, with increased progesterone production. The quantity of progesterone produced was related to the level of LH added. The cells were still steroidogenically capable and responsive to LH after storage for 48 hr at 4°.

The PMSG, LH, HCG, and prolactin used were supplied by the National Institutes of Health. The sheep were synchronized with the aid of Sil Estrus implants, a gift of Abbott Laboratories, North Chicago, Illinois. Ovulation was induced with Gonamone, a gift of Fort Dodge Laboratories, Fort Dodge, Iowa.

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Antiviral Activity of an Extract of *Brucella abortus*: Induction of Interferon and Immunopotentiation of Host Resistance[1] (39399)

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The induction of interferon in the virus-infected host has been utilized as one approach to antiviral chemotherapy. The most extensively investigated interferon inducer has been the polyribonucleotide complex, polyinosinic acid:polycytidylic acid [poly(I:C)]. Although poly(I:C) has been effective in a number of experimental infections in man and animals, important limitations have also been identified. The synthetic nucleotides induce relatively low levels of interferon in humans, and a variety of side effects have been identified in experimental animals (1). One alternative to the use of these synthetic compounds has been the development of natural biological products that have the capability of inducing interferon (2). Youngner and associates (3) have reported that an ether-extracted preparation of *Brucella abortus* (Bru-Pel) induced high levels of circulating interferon in mice, and protected animals against a subsequent lethal challenge of Semliki Forest virus. This report confirms the interferon-inducing capacity of Bru-Pel and extends the study of its potential as an antiviral agent to encephalomyocarditis (EMC) virus and *Herpesvirus hominis* type 2 (HVH-2) infections of mice. In each model, the efficacy of this compound as a biologic inducer is compared with poly(I:C), and its capacity to function as an immunopotentiator in enhancing host resistance to HVH-2 infections is described.

Materials and methods. Bru-Pel. This compound was prepared and supplied through the Antiviral Substances Program of NIH, by Jules Youngner, University of Pittsburgh, Pittsburgh, Pennsylvania. It was received as a lyophilized powder, and reconstituted in phosphate buffered saline (PBS) prior to inoculation into mice. Concentrations as high as 2000 μg per mouse did not produce any apparent toxicity.

Virus strains, media, cell cultures, and interferon assay. The origin and preparation of the MS strain of HVH type 2 and EMC virus pools, the media utilized for the maintenance of L-cell cultures, and the assay for interferon have all been described previously (4).

Statistical evaluation. To compare the final mortality of drug-treated and untreated mice, the data were evaluated by the Fisher Exact test.

Results. Interferon response to Bru-Pel. To determine the kinetics of the serum interferon response to Bru-Pel, groups of six 25-g female Swiss Webster mice were injected intraperitoneally (ip) with concentrations of 1000, 500, or 100 μg per mouse of Bru-Pel and pooled blood obtained at 3, 6, 9, 12, and 24 hr after injection. The capacity of Bru-Pel to induce interferon was compared with mice receiving 100 μg of poly(I:C) ip. The interferon titers obtained are presented in Table I. Peak interferon titers of 3000-5000 units were observed 6 hr after injection of each concentration of Bru-Pel, and persisted through 12 hr. At 24 hr only low levels were detectable. Similar levels of interferon were induced by poly(I:C), with the peak titer occurring at 9 hr after administration.

Hyporeactivity to the induction of interferon. One potential limitation to the use of interferon inducers is the decreasing interferon response observed after repeated injections (4, 5). To determine if Bru-Pel also induces such a state of hyporeactivity, groups of six animals each were given daily

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ons of 100 μg of Bru-Pel for 4 days ed at 3, 6, 9, 12, and 24 hr after each on. Interferon titers in serum from 6 ır in these animals are summarized in II. At 6-9 hr after the first injection, ınits of interferon were present, while he second dose of Bru-Pel the peak vas reduced to 600-900 units. After ird and fourth injections, only low (50-225 units) of interferon were pro- Although not shown in Table II, the ron levels in serum obtained at 3 and ıfter injection indicated that there was :t in the peak interferon response.

viral efficacy of Bru-Pel. Based on lata which demonstrate the capcity of bstance to induce interferon *in vivo,* a of experiments were designed to de- e the ability of Bru-Pel to protect ıgainst three different experimental ıfections: (i) 3-week-old mice infected ısally with HVH-2; (ii) adult mice in- ip with HVH-2; and (iii) adult mice d ip with EMC virus. The effect of :l or poly(I:C) on the mortality of 3- ›ld 10-15-g Swiss Webster mice in- intranasally with HVH-2 was evaluated by treating groups of animals with 500 μg of Bru-Pel or 100 μg of poly(I:C) once daily for 5 days beginning either 6 hr prior to, or immediately after, infection. In a representative experiment (Table III) the PBS-treated virus control animals had a final mortality of 70%. Mice that received therapy with Bru-Pel beginning 6 hr prior to infection had a reduced final mortality while those animals receiving treatment initiated immediately after infection did not. Mice treated with poly(I:C), however, had significantly lower final mortalities whether the treatment was begun prior to or after viral inoculation.

E I. INTERFERON TITERS IN SERUM OF ADULT CE INJECTED WITH Bru-Pel OR poly(I:C).

ron er use)	Interferon titers (units/ml) Hours postinjection				
	3	6	9	12	24
:l					
)	3075	4475	2150	3000	75
)	1100	3850	1075	2325	50
)	400	3225	1050	500	50
:C)					
)	3850	3750	5275	3400	150

LE II. INTERFERON TITERS IN SERUM FROM E GIVEN MULTIPLE INJECTIONS OF Bru-Pel.

Bru-Pel (100 lministration	Time of bleeding (hr)	Interferon titer (units/ml)
	6	4800
	9	4800
	12	1100
1	6	875
	9	600
	12	65
1, 2	6	50
	9	225
	12	50
1, 2, 3	6	50
	9	50

The effect of Bru-Pel or poly(I:C) on the mortality of adult mice infected ip with either EMC virus or HVH-2 was examined by treating groups of animals beginning either 6 hr prior to, or immediately after infection with 500 μg of Bru-Pel or 100 μg of poly(I:C) once daily for 10 days. Although treatment with Bru-Pel did reduce the mortality in EMC virus-infected mice (Table IV) therapy with poly(I:C) appeared to be superior [Bru-Pel compared with poly(I:C), $P < 0.01$]. Mortality in the two HVH-2 infected groups of mice treated with Bru-Pel was similar to control animals, while it was significantly reduced in the poly(I:C)-treated groups.

Nonspecific enhancement of resistance. It is recognized that bacterial preparations such as *Corynebacterium parvum (C. parvum)* and BCG have multiple effects on components of the host defense mechanisms. Previous studies from this laboratory indicated that mice pretreated (10-14 days) with a killed suspension of *C. acnes, C.*

TABLE III. EFFECT OF Bru-Pel ON THE MORTALITY OF 3-WEEK-OLD MICE INFECTED INTRANASALLY WITH HVH-2.

Treatment[a]	Mortality		P value
	Number	Percentage	
PBS	14/20	70	
Bru-Pel, −6 hr	6/20	30	<0.05
Bru-Pel, time 0	11/20	55	NS
Poly(I:C), −6 hr	4/20	20	<0.01
Poly(I:C), time 0	5/17	29	<0.05

[a] Treatment was initiated at either 6 hr prior to (−6 hr), or immediately after (time 0), infection.

TABLE IV. EFFECT OF Bru-Pel ON THE MORTALITY OF ADULT MICE INOCULATED IP WITH EMC VIRUS OR HVH-2.

Treatment[a]	EMC virus mortality: Number	EMC virus mortality: Percentage	P value	HVH-2 mortality: Number	HVH-2 mortality: Percentage	P value
PBS	15/15	100		14/15	93	
Bru-Pel, −6 hr	9/15	60	<0.05	12/15	80	NS
Bru-Pel, time 0	10/15	67	<0.05	14/15	93	NS
Poly(I:C), −6 hr	0/15	0	<0.001	2/15	13	<0.001
Poly(I:C), time 0	1/15	7	<0.001	2/14	14	<0.001

[a] Treatment was initiated at either 6 hr prior to (−6 hr), or immediately after (time 0), infection.

parvum, or BCG demonstrated enhanced resistance to infection with HVH-2 (6). These data suggested that Bru-Pel, a cell wall preparation of *Brucella abortus*, might also enhance resistance to viral infections of mice, independent of its capacity to induce interferon. To test this hypothesis, groups of 15 adult mice were treated ip with various concentrations of Bru-Pel 14 days prior to ip challenge with HVH-2. The results of one representative experiment are summarized in Table V. Concentrations of Bru-Pel ranging from 2000 to 125 μg/mouse were all effective in preventing mortality due to HVH-2 infection. In subsequent experiments, dosages of less than 125 μg were inactive.

TABLE V. NONSPECIFIC RESISTANCE TO HVH-2 INFECTION IN ADULT MICE INDUCED BY VARYING CONCENTRATIONS OF Bru-Pel.

Bru-Pel[a] (μg/mouse)	Mortality: Number	Mortality: Percentage	P value
None (PBS)	12/15	80	
2000	2/15	13	<0.001
1000	4/15	27	<0.01
500	1/15	7	<0.001
250	1/15	7	<0.001
125	2/15	13	<0.001

[a] Treatment ip 14 days prior to HVH-2 inoculation.

TABLE VI. NONSPECIFIC RESISTANCE TO HVH-2 INFECTION IN ADULT MICE INDUCED BY Bru-Pel ADMINISTERED AT VARIOUS TIMES PRIOR TO VIRAL CHALLENGE.

Treatment[a] (days)	Mortality: Number	Mortality: Percentage	P value
PBS, −14	13/15	87	
Bru-Pel, −1	4/15	27	<0.01
Bru-Pel, −4	12/15	80	NS
Bru-Pel, −7	7/15	47	0.05
Bru-Pel, −10	5/15	33	<0.01
Bru-Pel, −14	2/15	13	<0.001

[a] Treatment with 2000 μg was initiated 1–14 days prior to infection with HVH-2.

To determine the time interval necessary for induction of this nonspecific resistance, adult mice were given Bru-Pel 1, 4, 7, 10, and 14 days prior to ip inoculation of HVH-2. Control animals that received PBS 14 days prior to infection had a final mortality of 87% (Table VI). Animals receiving Bru-Pel 1 day prior to infection had a reduced final mortality presumably reflecting the action of interferon since no protection was observed when Bru-Pel was administered 4 days prior to infection. A significant degree of protection was observed, however, in mice receiving Bru-Pel 7, 10, and 14 days prior to infection as evidenced by final mortalities of 47, 33, and 13%, respectively. In these experiments both Bru-Pel and HVH-2 were administered by the ip route. This nonspecific enhancement of host resistance by Bru-Pel to HVH-2 infection may be the result of activation of macrophages within the peritoneal cavity. If this is the mechanism of protection one might expect that the effect would be compartmentalized, that is, confined to the peritoneal cavity. The next series of experiments were designed to determine whether this enhancement of host resistance to HVH-2 infection would occur when the virus was inoculated by a different route than the Bru-Pel. Groups of 2-week-old mice were initially injected ip with either PBS, killed *C. acnes*, or 1000 μg of Bru-Pel. Seven days later the mice were infected with HVH type 2 by either the intranasal or the ip route. The results from one experiment are tabulated in Table VII. There was no reduction of mortality in those animals pretreated with either *C. acnes* or Bru-Pel and

II. Nonspecific Resistance to HVH-2 Infection in 3-Week-Old Mice Induced by Bru-Pel.

-7	HVH-2 IN[b] Mortality			HVH-2 IP[c] Mortality		
	Number	Percentage	*P* value	Number	Percentage	*P* value
	25/38	66		18/39	46	
	21/31	68	NS	5/39	13	<0.01
	21/32	66	NS	3/33	9	<0.001

ys prior to infection with HVH-2.
culated intranasally.
culated intraperitoneally.

ed intranasally. In contrast, those
etreated ip with either compound
infected ip had a significantly
tality than control mice. These
st that both bacterial preparations
tive locally in the peritoneal cav-
t neither preparation was active
H-2 infection initiated by the in-
ute.

n. The kinetics of the interferon
o Bru-Pel is similar to that in-
iruses and synthetic nucleotides,
the more rapid production of
usually induced by bacterial
ch as endotoxin. The response of
acterial lipopolysaccharides is
ed by the induction of only a few
its of interferon with peak serum
rring at 1–3 hr after injection (2),
ru-Pel induced several thousand
a peak at 6–9 hr. The develop-
yporeactivity on the part of the
oduce interferon after multiple
of Bru-Pel is similar to that in-
oly(I:C) and tilorone hydrochlo-
. This progressive decrease in in-
oduction, however, may not nec-
an loss of antiviral activity since
et al. (7) have reported that
ses of poly(I:C) were more effec-
single dose in protecting mice
EMC virus infection.

u-Pel and poly(I:C) were more
ainst EMC virus than they were
H-2 infection in adult mice. This
may be due to the fact that EMC
o- to fourfold more sensitive to
of interferon than is HVH-2 (4).
ly, both inducers were more ef-
n HVH-2 was inoculated by the
an the intranasal route. We have
reported data which suggest that
asal inoculation, HVH-2 proba-
from the nasopharynx directly to the CNS (8) and thus may be less amenable to systemic therapy. This concept is supported by the evidence that chemotherapy with a number of antiviral agents has not been successful in preventing mortality due to HVH-2 infections initiated by the intranasal route, but was effective when the ip route of viral inoculation was used (4, 8–10).

In all three experimental viral infections utilized in this study, Bru-Pel did not appear to be as effective as poly(I:C). The reason for the diminished protective efficacy of Bru-Pel as an interferon inducer is not completely explained, particularly since the levels of interferon induced by a single dose of Bru-Pel are similar to those seen after a single dose of poly(I:C) and the degree of hyporeactivity associated with multiple doses of either drug is similar. These data suggest that the amount of interferon stimulated by interferon inducers should not be the only parameter measured to determine potential antiviral efficacy of such compounds. The data further suggest that host resistance mechanisms other than interferon may be stimulated by interferon inducers in the treatment of viral infections. Although the data presented suggest that Bru-Pel is less effective than poly(I:C) as an interferon inducer in protecting mice against viral infections, a number of factors should be considered in evaluating the potential of this substance for use in the treatment of viral diseases of man. In contrast to poly(I:C), it is possible that Bru-Pel will be an effective interferon inducer in humans and have little or no toxicity. Although preliminary evaluation suggests that Bru-Pel is considerably less toxic to mice than poly(I:C), detailed studies have not been carried out.

The enhancement of host resistance to HVH-2 infection when Bru-Pel was administered 10–14 days prior to viral inoculation suggests that this compound may have more than one mechanism of action. In support of this concept is the report of DeClercq and De Somer (11), who showed that administration of live *Brucella abortus* to rabbits 1 week prior to vaccinia virus challenge, inhibited lesion development in the absence of detectable circulating interferon. The results presented in this paper suggest that Bru-Pel, in addition to its action as an interferon inducer, can also protect mice from viral infection by nonspecific induction of resistance, presumably through the activation of cells of the reticuloendothelial system. Two other interferon inducers, poly(I:C) and tilorone hydrochloride, both failed to induce resistance to HVH-2 infection in mice when tested under similar conditions. The active component of Bru-Pel responsible for nonspecific enhanced resistance is not known. It does not appear to be mediated by endotoxin, however, since *E. coli* 0111.B4 lipopolysaccharide (Difco), or a glycolipid preparation from *Salmonella minnesota* failed to induce enhanced resistance to HVH-2 infection of mice (unpublished results). The stimulation of an exudative reaction of cells in the peritoneal cavity does not provide an adequate explanation either, since three inducers of an inflammatory reaction (peptone, glycogen, and thioglycollate) used in our laboratory have failed to increase nonspecific resistance to HVH-2 infection in mice using pretreatment regimens, even though all stimulated a cellular reaction in the peritoneal cavity.

Summary. These data provide evidence that (i) the peak level and duration of the serum interferon response induced in mice by Bru-Pel is similar to that induced by some viruses and poly(I:C); (ii) the induction of interferon is markedly reduced after multiple injections of Bru-Pel; (iii) when administered as an interferon inducer, Bru-Pel does not appear to be as effective as poly(I:C) in protecting mice infected with either encephalomyocarditis virus or *Herpesvirus hominis* type 2; and (iv) when given 10–14 days prior to virus inoculation, Bru-Pel is effective as a nonspecific enhancer of host resistance to viral infection. These data suggest that Bru-Pel, in addition to its use as an interferon inducer, may have a role as a nonspecific potentiator of host resistance similar to that which has been reported for other bacterial agents such as *C. parvum* and BCG.

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Absorption of Heparin and Cyanocobalamin from the Rat Lung[1] (39400)

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The respiratory tract is a frequently used site for the administration of a variety of volatile and nonvolatile therapeutic agents (1, 2). In the case of nonvolatile drugs, the intention of inhalation therapy has usually been that the compounds act locally within the lungs. However, in a few instances, nonvolatile agents have been administered into the respiratory tract with the intention that they exert a systemic action. For example, heparin, a substance that is poorly absorbed from the gastrointestinal tract and oral cavity (3-6), has been shown to produce a systemic anticoagulant effect when inhaled as an aerosol by normal human subjects (7). Moreover, cyanocobalamin (vitamin B_{12}), a substance which requires the presence of intrinsic factor for rapid intestinal absorption and which is very poorly absorbed from the gastrointestinal tract in the absence of intrinsic factor (8), has been shown to be therapeutically effective in the treatment of pernicious anemia when administered to patients by inhalation of powders or liquid aerosols containing the vitamin (9, 10).

From the work cited above, it is not clear whether absorption of inhaled heparin and cyanocobalamin occurs by diffusion or by a more complex transport process. Moreover, the studies provide no information on the rate of absorption of these substances from the respiratory tract. In the present investigation in the rat, quantitative evidence is presented that describes the mechanisms and relative rates of pulmonary absorption of heparin and cyanocobalamin.

Materials and methods. Procedure in animals. To investigate the absorption of heparin and cyanocobalamin from the lung, male Charles River-derived rats (150-200 g) were anesthetized with pentobarbital (50 mg/kg) and prepared surgically according to a method described previously (11). Briefly, the trachea was exposed through a ventral midline incision in the neck. A 2.5-cm length of PE 240 tubing, which served as a tight-fitting tracheal cannula, was inserted through an incision between the fourth and fifth tracheal rings caudal to the thyroid cartilage to a depth of 0.6 cm. Solutions of heparin (0.001-1 m*M*) or cyanocobalamin (0.01-1 m*M*) were prepared by adding radioactively labeled compound together with unlabeled compound to Krebs-Ringer phosphate solution (pH 7.4), in which Ca ion had been lowered to one-fifth the usual concentration to avoid turbidity (12). One-tenth milliliter of solution was injected into the lungs through PE 20 tubing attached to a calibrated 100-μl syringe. The injection tubing was inserted through the tracheal cannula to a point approximately 1 mm above the bifurcation of the trachea, the solution injected over a 1-2 sec interval, and the tubing quickly withdrawn. The incision in the skin was then closed with a wound clip and body temperature maintained at 37 ± 1° by heat from a 40-W incandescent lamp suspended above the animal.

Tissue extraction and drug assay. At the end of an absorption period (0.25-12 hr), the lungs, with attached trachea and cannula, were excised from the animal. The tissue was weighed and placed in a 15-ml Tenbroeck glass homogenizer together with washings from the tracheal cannula and sufficient distilled water to make a total weight of 4 g. After homogenization, 100-mg samples of the tissue homogenate were transferred into glass liquid scintillation counting vials, and to each vial was added 0.2 ml of 60% perchloric acid together with 0.4 ml of 30% hydrogen peroxide. The vials were then heated at 70° for 1-1.5 hr. To the resulting tissue digest was added 20 ml of a

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liquid scintillation medium of the following composition: 6 g of 2,5-diphenyloxazole, 573 ml of toluene, and 427 ml of ethylene glycol monomethyl ether. Radioactivity was measured using a Packard model 3375 Tri-Carb liquid scintillation spectrometer equipped with automatic external standardization. Net counts per minute were corrected for quench by comparison with a standard quench correlation curve determined for the liquid scintillation medium used. It was assumed that no radioactivity was lost by animal expiration. When known amounts of compound were added to lung tissue and the assays carried out as described above, recoveries (± SE in three determinations) were essentially complete: heparin 99 ± 1% and cyanocobalamin 98 ± 2%.

Diffusion coefficients. For the following compounds, coefficients of free diffusion (water, 37°), as measured by the method of Schantz and Lauffer (13), have been reported previously: mannitol, sucrose, inulin, and dextran (14); erythritol (11); and urea (15). Diffusion coefficients for sulfanilic acid, *p*-aminohippuric acid (PAH), tetraethylammonium (TEA), procaine amide ethobromide cation (PAEB), and *p*-acetylaminohippuric acid (PAAH) were calculated previously (12) from the Stokes-Einstein equation (16, 17). Diffusion coefficients for heparin and cyanocobalamin were calculated in the present study from the Stokes-Einstein equation, and densities used in the calculations were determined by air displacement using a Fekrumeter (Gallard-Schlesinger Co.).

Drugs. Radioactively labeled [^{35}S]heparin, specific activity 3.23 mCi/g (64.6 mCi/mmole based on a mol wt of 20,000), and [G-^{3}H]cyanocobalamin, specific activity 1.65 Ci/mmole, were obtained from Amersham-Searle. Unlabeled heparin sodium was obtained from Fisher Scientific Co., and cyanocobalamin from Sigma Chemical Co. With the above radioactively labeled compounds, it has been shown by the manufacturer using electrophoresis that the labeled heparin has a radiochemical purity greater than 95% and the labeled cyanocobalamin a radiochemical purity greater than 98%.

Results and discussion. Results of pulmonary absorption studies with heparin and cyanocobalamin are shown in Fig. 1. When the percentage of unabsorbed radioactivity was plotted semilogarithmically against time, the data for each compound conformed to a straight line, suggesting first-order kinetics for the absorption process. Half-times for absorption and apparent first-order rate constants, calculated from the slopes of the lines, are listed in Table I. It can be seen that the time required for 50% of a dose to be absorbed was 3 hr for cyanocobalamin and 9.2 hr for heparin.

Evidence that both compounds are absorbed by a nonsaturable transport process, such as simple diffusion, was seen when absorption rates were measured over a wide range of initial concentrations. As shown in Table II, when the concentration of cyanocobalamin was varied 100-fold, from 0.01 to 1 m*M*, the 2-hr absorption rate was directly proportional to concentration, the percentage absorption remaining essentially constant. Similarly, concentration studies with 0.001-1 m*M* heparin showed no evidence of saturation over the 1000-fold

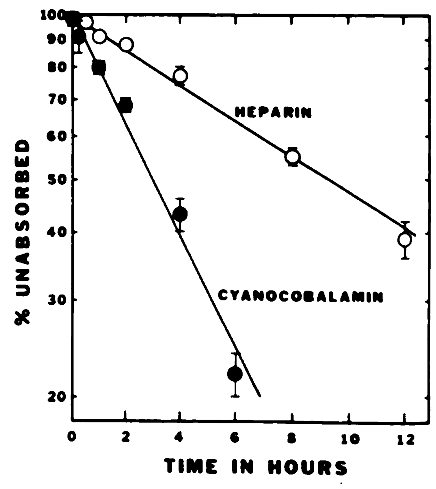

FIG. 1. Absorption of radioactivity from the rat lung after administration of [^{35}S]heparin and [^{3}H]cyanocobalamin. Krebs-Ringer phosphate solution (0.1 ml), containing a compound (0.1 m*M*), was administered intratracheally to anesthetized rats prepared with a tight-fitting tracheal cannula. Each point is the mean of three to six animals. Vertical brackets indicate SE, and absence of brackets indicates that SE was too small to be shown.

:ABLE I. ABSORPTION OF RADIOACTIVITY FROM THE RAT LUNG AFTER ADMINISTRATION OF [^{3}H]CYANOCOBALAMIN AND [^{35}S]HEPARIN.

			Rate of absorption[a]	
npound	Mol wt	Diffusion coefficient (D, 37°) cm^2/sec × 10^6	Half-time (hr)	Rate constant × hr^{-1}
cobalamin	1,355	4.33	3.0	0.231
in	6,000-20,000	2.87-1.93[b]	9.2	0.0753

ent first-order rate constant is equal to 0.693 divided by the half-time in hours. Half-time values were rom the curves shown in Fig. 1.

on a molecular weight range of 6,000-20,000 (18, 20, 21).

E II. EFFECT OF CONCENTRATION ON ABSORPTION OF RADIOACTIVITY FROM THE RAT LUNG AFTER ADMINISTRATION OF [^{3}H]CYANOCOBALAMIN AND [^{35}S]HEPARIN.

		Percentage of dose absorbed[a]			
		Concentration (mM)			
mpound	Time for absorption (hr)	0.001	0.01	0.1	1.0
cobalamin	2		35 ± 1	33 ± 2	39 ± 3
in	8	43 ± 4		45 ± 2	46 ± 2

Ringer phosphate solution (0.1 ml), containing various concentrations of a compound, was adminis- racheally to anesthetized rats prepared with a tracheal cannula. Each absorption value is the mean of ls ± SE.

ince percentage absorption re-
onstant at 43-46% (Table II).
us work in this laboratory (11, 12)
sted that the respiratory tract epi-
f the rat has permeability charac-
imilar to those of the classical li-
type of biologic membrane. Li-
ble compounds are absorbed more
han lipoid-insoluble compounds,
rates of absorption are generally
the lipid/water partition coeffi-
the compounds. Lipid-insoluble
s appear to be absorbed primarily
cted diffusion through aqueous
the pulmonary membrane at rates
related to their molecular size.
r, a comparison of pulmonary ab-
ates (apparent first-order rate con-
with diffusion coefficients (D, in
7°) for urea and some saccharides
that there is at least three differ-
of pores in the absorbing mem-
). Since both cyanocobalamin and
are lipoid-insoluble compounds
nd would accordingly depend pri-
the pore route for their absorp-
of interest to compare their k and
with similar values obtained previ-
certain other lipoid-insoluble mol-
widely different molecular size. In
alues reported previously (11, 12)

for several compounds of molecular weight 60 to 75,000 are plotted semilogarithmically against D values for the same compounds. From these data, it should be possible to predict with some degree of accuracy the pulmonary absorption rate for most other lipoid-insoluble compounds that are absorbed mainly by diffusion through membrane pores. When k and D values for heparin and cyanocobalamin are plotted on this figure, it can be seen that the points for both compounds appear on or very near the drawn curve. Thus, the observed absorption rates for heparin and cyanocobalamin are about what would be predicted on the basis of their calculated diffusion coefficients.

Summary. Solutions of heparin and cyanocobalamin were administered through a tight-fitting tracheal cannula into the lungs of anesthetized rats. The times necessary for 50% absorption from the lungs were 3 hr for cyanocobalamin and 9.2 hr for heparin. The absorption process did not become saturated when drug concentrations were raised 100 to 1000-fold, suggesting that both compounds are absorbed mainly by a process of simple diffusion. A comparison of pulmonary absorption rates and diffusion coefficients of heparin and cyanocobalamin with corresponding values reported previously for other lipoid-insoluble compounds

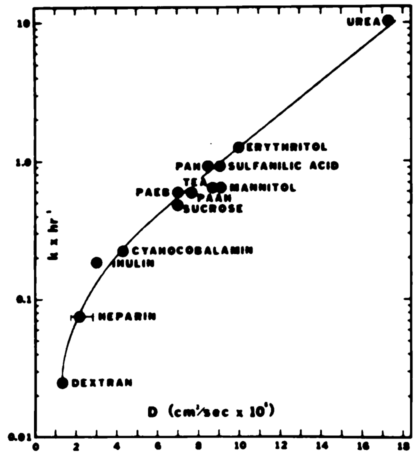

FIG. 2. Comparison of pulmonary absorption rate constants ($k \times hr^{-1}$) with diffusion coefficients (D, $cm^2/sec \times 10^6$) for various lipoid-insoluble compounds. Rate constants for all compounds were measured in this laboratory and, except for heparin and cyanocobalamin, have been reported previously (11, 12). Rate constants for the latter two drugs are taken from Table I. Diffusion coefficients for the compounds were either measured or calculated (see Materials and Methods section for details). The diffusion coefficient for heparin was calculated for a molecular weight of 13,000, and horizontal brackets indicate values calculated for a molecular weight range of 6,000-20,000 (18, 20, 21). An estimated curve of best fit has been drawn through the points.

indicated that observed absorption rates were close to the rates predicted for the two drugs based on their calculated diffusion coefficients and the view that absorption occurs mainly through membrane pores.

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I OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 381-384 (1976)

oral Variations in Reproductive Hormones in the Immature Male Rat (39401)

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(Introduced by R. L. Clancy)

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hmical changes in serum levels of ies are well known (1). In the adult t a daily maximum in luteinizing hor- LH) and prolactin has been reported ır at about the middle of the dark (2); another study showed the peak actin at the beginning of the dark (3). In the human male the highest evels of LH (4) and testosterone (5) en found late in the dark or early in t period. A daily peak in serum tes- ie for the adult male rat was found at which was also late in the dark period the other hand, rhythmical changes n follicle stimulating hormone (FSH) en difficult to establish; one study he male rat failed to show a rhythm nother (8) reported a peak in serum 0400 or 2 hr before the start of a -hr light period.

ntrast to the situation in adults, ss information is available on the al changes in hormones of immature . The concept is generally accepted thms develop with age and become hed only at about the time of pu-). Prominent nocturnal increases in H (10) and testosterone (11) have ported for the pubertal human male. prepubertal male rat (35 day) in FSH or LH were not found (8). k of information regarding variations onal levels in the post weaning male npted the present study; the results stinct changes with time.

ials and methods. Male rats of the an strain were used. They were in temperature (24 ± 2°) and light light: 0600–2000) controlled quar- h free access to food and tap water. vere opened and each animal han- ily prior to the beginning of the group of five animals was decapitated every 4 hr from 0800 on the twenty-fifth day until 1600 on the twenty-sixth day of age. Decapitation was done as soon as possible (5–15 sec) after the animal was removed from the cage. Since stress may alter hormonal patterns (2), efforts were made to minimize disturbance during this procedure.

Serum was separated by centrifugation (1500 *g* for 15 min) after the blood had clotted at room temperature; it was stored at −17°. Assays were performed in the following sequence: (i) LH(homologous system); (ii) FSH; (iii) testosterone; (iv) androstenedione(androst-4-ene-3,17 dione); (v) LH (heterologous system); (vi) 20α-dihydroprogesterone (20α-hydroxypregn-4-en-3-one) (20αDHP); and (vii) progesterone. If there was insufficient volume of serum for the next determination the sample was dropped from the study.

LH and FSH were measured by a double antibody radioimmunoassay using materials supplied by the NIAMDD-Rat Pituitary Program. LH was also assayed using anti-ovine LH (prepared by Dr. Gordon Niswender, Colorado State University) and iodinated ovine LH (NIAMDD). The details of the assay procedures have been published (12). The results of all assays were expressed in terms of rat standards (RP-1 LH; RP-1 FSH).

The steroids were also measured by radioimmunoassay. Anti-testosterone and anti-progesterone were prepared in this laboratory and their characteristics in assays have been reported (13). Anti-androstenedione was a gift of Drs. H. Lindner and S. Bauminger of the Weizmann Institute (Rehovot, Israel); its use in assays of this steroid have been published (14). Anti-20αDHP was supplied by Drs. C. N. Pang and J. Hilliard (University of California, Los An-

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rved.

geles). The 20αDHP was attached through the carbon-3 position to bovine serum albumin for an antigen. The only steroid which gave appreciable cross-reactivity (>1%) with the antiserum was 20βDHP (42%).

Tritiated steroids were purchased from New England Nuclear Corp (Boston, Mass). Specific activities were: testosterone 45 Ci/mmole; androstenedione 48 Ci/mmole; progesterone 50.3 Ci/mmole; 20 DHP 50.3 Ci/mmole. Steroid standards were obtained from Sigma Chemical Co. (St. Louis, Mo.) and were dissolved in methanol.

The steroid assays were performed in phosphate buffered saline (0.1 *M*, pH 7.0). Serum (25-50 μl) was diluted with 1 ml of distilled water and extracted once with 3 volumes of freshly opened diethyl ether. Extraction efficiency was evaluated by extraction of 5000 cpm of steroid from 1 ml of water. The ether extracts were taken to dryness and redissolved in 0.1 ml of buffer containing 0.1% gelatin. Sufficient antibody to bind 40-55% of the labeled steroid was added and the solution incubated at room temperature for 30 min. After addition of 15,000 dpm of labeled steroid, the incubation was continued overnight at 4°. Free and antibody-bound steroids were separated by dextran-coated charcoal and centrifugation for 10 min at 1500 *g*. A 0.5-ml aliquot of the supernatant was placed in a scintillation vial with 5 ml of Bray's solution and counted for 10 min in a Packard scintillation spectrometer (35% efficiency for tritium).

Standard curves for gonadotropin and steroid assays were linearized by logit and log dose transformations; unknowns were extrapolated after linear regression analysis. Samples were randomized and analyzed in duplicate for a particular hormone in a single radioimmunoassay. Intraassay coefficients of variation between duplicates did not exceed 10%. Values below the sensitivity of the assay were not included in statistical calculations. Data were analyzed by one-way analysis of variance and by Duncan's new multiple range test (15), with a probability of less than 5% considered statistically significant.

Results and discussion. The results of the study are shown in Fig. 1. Serum FSH concentrations changed significantly with time [$F(8,29) = 2.66$, $P < 0.05$]. It was high in the morning (0800), lower during the remainder of the light period (1200-1600), and then steadily increased to a high point near the end of the dark period (0400). A very similar pattern was found previously in adult males of the same strain housed under the same conditions (8). Serum testosterone [$F(8,35) = 3.09$, $P < 0.01$] and androstenedione [$F(8, 35) = 3.39$, $P < 0.01$] also had significant variations with time; the androgens were correlated with each other ($r = 0.95$, $P < 0.01$).

Testosterone showed the same degree of variation and the same changes with time as has been reported for the adult male rat (6). In the latter study serial bleedings from animals under ether anesthesia were used: ether is a stress that can alter endocrine rhythms (2). Although the serum androgens followed the same general pattern of changes as did FSH, comparison of individual serum samples shows that there is not a significant correlation (FSH:testosterone, $r = 0.27$) (FSH:androstenedione, $r = 0.26$). However, the recent finding that highly purified FSH is as effective as LH in raising serum testosterone in hypophysectomized male rats (16) indicates that we must consider the possibility that the FSH was the stimulus for the increases in androgen.

Serum LH[$F(8,33) = 1.17$, NS], progesterone [$F(8,25) = 1.74$, NS], and 20αDHP [$F(7,27) = 1.09$, NS] did not significantly vary with time. LH was highest at 0800 and 0400 but large differences between animals within groups prevented statistical significance. The results for LH were confirmed (data not shown) in a heterologous ovine radioimmunoassay system which is more sensitive than the homologous assay. Comparison of LH levels with those of testosterone in the same samples failed to show a significant correlation ($r = 0.15$), but LH and FSH are correlated ($r = 0.51$, $P < 0.01$).

The demonstration of a distinct rhythm in serum androgens without significant changes in LH suggests lack of a relationship. However, we measured a rapidly disappearing gonadotropin at infrequent intervals and therefore short periods of increased output of LH by the pituitary could easily be missed. There is the possibility that rhythmical changes in Leydig cell steroidogenic

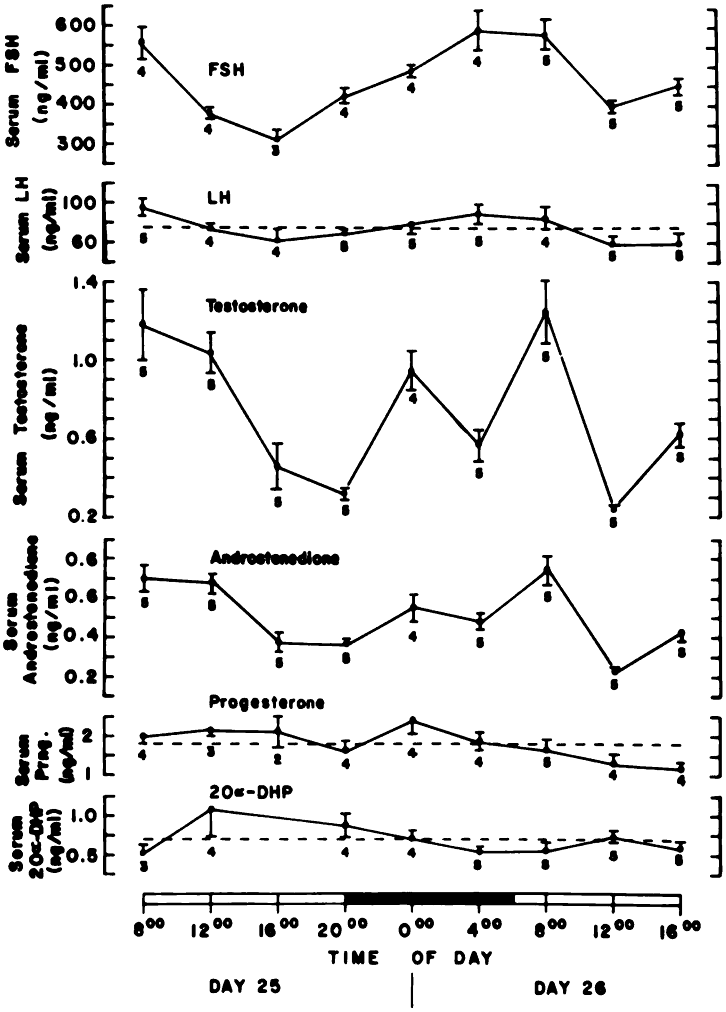

FIG. 1. Variations with time in serum gonadotropin and steroid concentrations in male rats. Groups were decapitated at 4-hr intervals on the twenty-fifth and twenty-sixth days of life. Vertical lines indicate standard errors for the number of observations indicated. The dashed line denotes the grand mean when variations with time were insignificant. Shaded area indicates daily dark period.

function occur independently from changes in gonadotropin. Previous studies using hypophysectomized immature male rats treated with LH and prolactin suggested that the testes were more sensitive to gonadotropic stimulation during the dark period (17). A similar suggestion has been proposed to account for the diurnal variation in testosterone in the human (11). Preliminary experiments (D. C. Johnson and H. C. Cheng, unpublished) have shown that increased testosterone levels are maintained longer in hypophysectomized males given LH and prolactin in the dark period compared to those injected in the light period; the magnitude of the increase in androgen was not time dependent, only the duration. These results could also be interpreted as indicating fluctuations in the rate of destruction of hormone, rather than in the rate of secretion. This aspect has been inadequately studied but in the human at least the metabolic clearance rate of testosterone does not change with time (5).

The significance of the changes with time found in the present study are not clear. The pattern of serum concentrations needs to be followed for a much longer time in order to establish whether a true circadian rhythm (20-28 hr) is involved. These data do clearly demonstrate, however, that studies of gonadotropins and androgens in immature male rats must consider temporal changes. The absolute values for the serum androgens in the immature rats were relatively low but the temporal fluctuations for testosterone represent a fivefold and for androstenedione a threefold variation in mean concentrations.

Summary. Radioimmunoassays were used to measure serum levels of LH, FSH, testosterone, androstenedione, progesterone, and 20αdihydroprogesterone in immature (25-26 day) male rats decapitated at various times of the day. Only FSH, testosterone, and androstenedione showed a significant variation with time; the maximal values occurred late in the dark and early in the light period.

We thank the NIAMDD-Rat Pituitary Program for generous supplies of gonadotropins used in the immunoassays, and Dr. Gordon Niswender for anti-ovine LH. This research was supported in part by a grant from the National Science Foundation (BMS 74-16360). H. E. G. was a predoctorate fellow (NIH-HD-00025).

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:tural Changes in Glyceraldehyde-3-Phosphate Dehydrogenase during Temperature Acclimation of Rainbow Trout (39402)

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ıt reports it has been shown by us
d others (3) that the kinetics of
solated from members of a species
been acclimated to different tem-
have been altered. In addition, it
reported by us that the changed
)perties may be the result of modi-
ry or quaternary structure of the
l). In the present report, immuno-
:hniques have been used to dem-
ı change in the conformation of
hyde-3-phosphate dehydrogenase
', isolated from rainbow trout ac-
o 5 and 15°.

ls and methods. Rainbow trout,
ırdneri, were obtained from the
States Fish Disease Laboratory,
West Virginia. One group was
l at 15°, the other at 5°, using an
:ean Aquarium System. The tem-
were maintained ± 2°. The length
acclimation was 3 weeks for both

tion of G3PDH was carried out
to the method of Cori *et al.* (5) as
in our laboratory by Wolfe (11).
15° (warm enzyme) and 5° (cold
33PDH were isolated at 5°. Crys-
PDH which gave a single protein
rylamide electrophoresis were ob-
.e crystals were separated from the
|uor by centrifugation at 30,000 *g*,
solved in triethanolamine buffer,
The protein concentrations were
d by the procedure of Lowry (6)
absorbance at 260 and 280 nm.
nes were stored at pH 8.4 at 5°
ddition of dithiothreitol.

male New Zealand rabbits were
l with the purified enzymes, using
adjuvant for the initial injection
ım adjuvant for the following in-
l ml/mg enzyme). Intramuscular
into the hindlimb near the poplit-
were used. Two time courses of
immunization were followed: a short-term (ST) course and a long-term (LT) course. The short-term course consisted of an initial injection of 0.25 mg of enzyme, an injection of 0.50 mg 30 days later, and three injections of 1.00 mg of enzyme 4, 3, and 4 days, respectively, after the previous injections, for a total of 15 days from initial to final injection. Five days after the last injection, cardiac punctures of 20 ml were done on each rabbit as a source of ST serum for further tests. Injections were resumed 2 days later (the rabbits not having been injected for 1 week) and succeeding injections of 1.0 mg of enzyme once per week continued for 9 more weeks as an LT course of hyperimmunization. The rabbits were bled by cardiac puncture 5 days after the last injection. The serum was separated, divided into 1-ml aliquots and frozen at −10° until used. Once thawed, serum was not refrozen.

G3PDH was assayed by measuring the reduction of NAD at 340 nm using G3P and arsenate as substrates in pyrophosphate buffer at pH 8.4. The 3-ml cuvette in a Beckman, DBG recording spectrophotometer was used and the reaction initiated by the addition of 2 μg of enzyme. The reaction mixture contained 40 m*M* triethanolamine, pH 8.4, 17 m*M* Na_2HAsO_4, 1.58 m*M* NAD, and G3P from 18.3 to 300 μ*M*.

The K_m and V_{max} were determined by least Square Regression of the Lineweaver and Burke plots of the data using computer programs in the Computer Center Library.

Results and discussion. The results of the immunological tests are represented in Figs. 1 and 2. Interfacial microprecipitin tests (7) performed on the short-term (ST) antisera were specific for each enzyme, 5 and 15°, with no cross section. Precipitin tests on the long-term (LT) antisera showed limited cross reaction with both enzymes. Ouchterlony immuno-double diffusion studies (7, 8)

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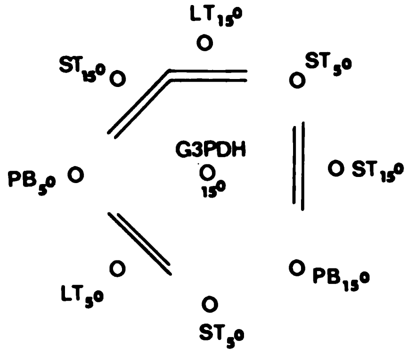


FIG. 1. Results of interfacial microprecipitin tests. G3PDH 15°, enzyme isolated from rainbow trout acclimated to 15° and used as antigen; $ST_{15°}$, $LT_{15°}$, $ST_{5°}$, $LT_{5°}$, short-term and long-term antisera to 15° and 5°, respectively; $PB_{15°}$, $PB_{5°}$, sera obtained from rabbits prior to immunization with enzyme obtained from 15 and 5° acclimated rainbow trout.

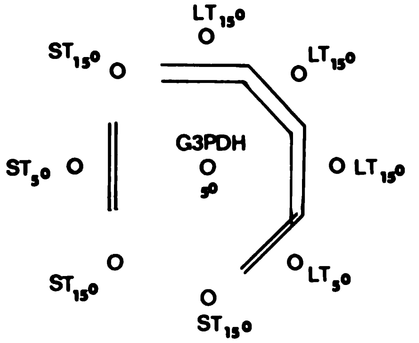


FIG. 2. Results of interfacial microprecipitin tests. G3PDH 5°, enzyme isolated from rainbow trout acclimated to 5° and used as antigen. Other descriptors as in Fig. 1.

confirmed these results. Reactions of identity were found between ST and LT antisera to cold enzyme when reacted with the cold enzyme, and also between ST and LT antisera to warm enzyme when reacted with the warm enzyme. However, reactions of nonidentity were found between LT antiserum to warm enzyme and LT antiserum to cold enzyme when reacted with cold enzyme, as well as between LT antiserum to warm enzyme and LT antiserum to cold enzyme when reacted with the warm enzyme. Thus, the LT antisera did cross react, but were distinctly different. Since antibody production is dependent on tertiary conformation, it appears that there is a difference in the tertiary structure of each enzyme. These results support the previous reports from our laboratories (4) suggesting an *in vivo* change in conformation during temperature acclimation.

Polyacrylamide gel electrophoresis of the two enzymes gave single protein bands in each case. When cochromatographed, polyacrylamide gel electrophoresis failed to separate the enzymes, using three different gel cross-linkages.

The kinetics of the enzymes in the presence of antisera were then investigated. As controls, serum of the rabbits, prior to immunization (PB), and serum of rabbits not immunized (NI) were incubated with the enzyme for 5-min before assay. No measurable effect was seen on the kinetics of the reactions when reacted with either control serum. When specific antisera were incubated with the enzymes, for the same length of time, it was found that the kinetics of the enzymes were markedly affected. A Lineweaver–Burke analysis of the data indicated an irreversible change (Table I and Figs. 3 and 4).

The cold enzyme, when reacted with PB or NI serum, yielded a control K_m of 116 μM and a V_{max} of 0.238 μmole/min/mg. When reacted with ST antiserum to cold enzyme, the K_m was unchanged by V_{max} was reduced to 0.066 μmole/min/mg, demonstrating marked inhibition. With LT anti-

TABLE I. REACTION KINETICS OF G3PDH AS AFFECTED BY ANTISERA TO "ACCLIMATED" ENZYMES.[a]

	Test enzyme			
	Warm (15°)		Cold (5°)	
Antiserum to	K_m[b]	V_{max}[c]	K_m	V_{max}
Control	208	3.4	116	0.238
Warm enzyme ST	208	0.83	116	0.201
Warm enzyme LT	208	0.84	116	0.192
Cold enzyme ST	208	3.3	116	0.066
Cold enzyme LT	208	2.5	116	0.078

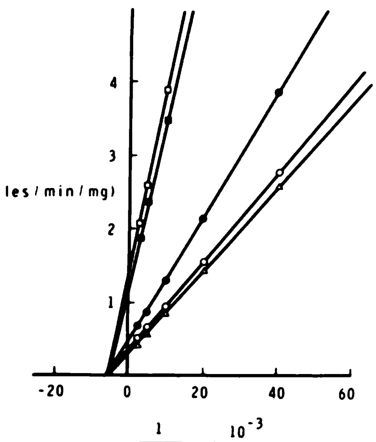
[a] The standard deviation of the calculated values are ± 15% of the above value taken from the Lineweaver-Burke plots.

[b] K_m in μM.

[c] V_{max} in μmole/min/mg.

cold enzyme, the same K_m and a 0.078 μmol/min/mg resulted. While istically different from each other, erimental results are different from ($P < 0.05$). Reaction of the cold with the ST antiserum to warm en- elded the same K_m and a V_{max} of mole/min/mg, results not different e control assay. However, reaction LT antiserum to warm enzyme the same K_m but a V_{max} Of 0.192 nin/mg, indicating only limited inhi- the reaction (not statistically signif-

tatively, similar results were ob- ith the assays of the warm enzyme. is enzyme was incubated with NI K_m of 208 μM and a V_{max} of 3.4 nin/mg were obtained. With ST an- to warm enzyme, the same K_m, but of 0.83 μmole/min/mg resulted, rating significant inhibition ($P <$ eaction with the LT antiserum to DH yielded the same K_m and a V_{max} mole/min/mg. Incubation with the erum to cold enzyme yielded the and a V_{max} of 3.3 μmole/min/mg, rent from controls. However, incubation with the LT antiserum to cold enzyme yielded the same K_m, but a V_{max} of 2.5 μmole/min/mg, indicating some limited degree of inhibition.



Lineweaver-Burke analysis of control ctivity. The 15° enzyme has been allowed to ST (□) or LT (■) antiserum to 15° G3PDH, or LT (●) antiserum to 5° G3PDH, or PB or) serum. K_m and V_{max} values with respect to calculated by iterative curve fitting using east squares. The standard deviation of the respect to the calculated lines is less than

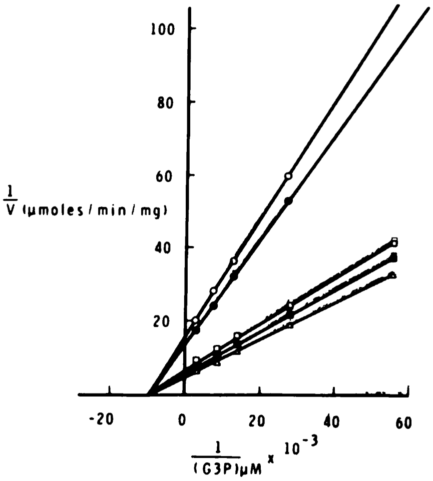


FIG. 4. Lineweaver-Burke analysis of cold acclimated G3PDH activity. The 5° enzyme has been allowed to react with ST (○) or LT (●) antiserum to 5° G3PDH, or ST (□) or LT (■) antiserum to 15° G3PDH, or PB or control (△) serum. K_m and V_{max} values with respect to G3PDH were calculated by iterative curve fitting using weighted least squares. The standard deviation of the points with respect to the calculated lines is less than ±15%.

Each antiserum reacted primarily in an irreversible manner with its specific enzyme. The degree of cross reactivity seen with the LT antisera indicates that the structures of the enzymes have areas of similarity. However, the kinetic data indicate that the enzymes are quite different in activity. The values obtained for the K_m and V_{max} of each enzyme are in agreement with those of Wolfe and Gray (4), the 5° enzyme having a greater affinity for the substrate, but a lower V_{max} than the 15° enzyme. The subunits of the G3PDH molecule are probably identical in composition (9) with differing *in vivo* conformations in the two altered environments. Antibodies formed to these enzymes were capable of distinguishing them, indicating such a structural change. These alternate forms of the enzyme are also quite stable, retaining their acquired differences through the purification process.

It would seem that this multimeric enzyme, composed of identical subunits, is capable of achieving different conformations as a result of chronic exposure to altered environmental temperature. This could result in changed NAD^+ binding to cause the different activities observed. Perhaps the actual mechanism of the altered enzyme conformation is dependent upon another temperature-dependent metabolic process, e.g., one leading to differences in *in vivo* salt concentrations at different temperatures. Still, our data do not rule out the possibility that the subunits of the acclimated enzymes are actually different, and that current methods have been unsuccessful in distinguishing them. Such changes in subunit structure would also incite different antibody production. However, the fact that long-term antibodies gave some cross reactions *in vitro* indicates that the subunit differences may be quite subtle. One might suggest new protein synthesis in cold acclimation as a result of inducer–repressor mechanisms with temperature change, where a gene capable of producing an enzyme with but slightly different subunit amino acid sequence, becomes turned on in the cold. Such gene-related changes may be mainly the result of changes in the concentration of cell metabolites and ions previously reported (10, 11).

Summary. G3PDH was isolated from rainbow trout acclimated to 15 and 5°. Antisera to each of the enzymes were prepared in rabbits. Microprecipitin tests showed that the short-term (ST) antiserum reacted only with its specific antigen. However, with the long-term (LT) antisera, there was some cross reaction. Ouchterlony double diffusion confirmed these results. Kinetic studies of each of the G3PDH enzymes in the presence of its specific antiserum were carried out. Specific ST and LT antisera significantly inhibited the activity of the enzyme. No significant effect was seen with the antisera to the noncomplementary enzyme. Some cross reaction was seen with the LT antisera in both instances. The Lineweaver-Burke analyses demonstrated no change in the K_m while the V_{max} was reduced, indicative of noncompetitive inhibition by the specific antisera.

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Failure of Intrahypothalamic Implants of an Estrogen Antagonist, moxytriphetol (MER-25), to Block Neonatal Androgen-Sterilization[1] (39403)

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well documented that androgen ad-
ation to neonatal female rats evokes
characterized by polyfollicular ano-
y ovaries and persistent vaginal cor-
on (neonatal androgenization; 1-4).
ly, several workers have postulated
conversion of androgen to estrogen
erequisite for induction of neonatal
enization (5-10). One of the reports
ting this postulation is that subcuta-
injection of an estrogen antagonist,
xytriphetol (MER-25), 6 hr prior to
rone propionate (TP), could block
enization (8). However, Brown-
11) reported that he obtained only a
us influence of the estrogen antago-
induction of neonatal androgeniza-
he present author has also failed to
a blocking effect of MER-25 on
l androgenization (12). In the pres-
es of the experiments, although the
of MER-25 per pellet was increased
and that of TP was reduced to threshold level for induction of anovulatory sterility, the ineffectiveness of MER-25 on neonatal androgenization was confirmed.

Materials and methods. Fifty-eight female rats of the Sprague–Dawley strain were divided into six groups at 4 days of age (day of birth = Day 1). Group I received a subcutaneous injection of 0.02 ml of sesame oil at 5 days of age (8 rats); group II received a subcutaneous injection of 30 μg of TP at 5 days of age (5 rats); group III received bilateral implantation of intrahypothalamic paraffin micropellets (sham operation) at 4 days of age and a subcutaneous oil injection (7 rats); group IV received the same operation as group III (sham) and a subcutaneous injection of 30 μg of TP at 5 days of age (12 rats); group V received intrahypothalamic micropellets of MER-25-paraffin mixture at 4 days of age and a subcutaneous oil injection at 5 days of age (11 rats); and group VI received the same implants as group V but followed by a subcutaneous injection of 30 μg of TP at 5 days of age (15 rats).

I. MEANS (±SE) OF DAY OF VAGINAL OPENING (VO) AND ONSET OF PERSISTENT VAGINAL ESTRUS AND INCIDENCE OF STERILITY (IS) IN RATS RECEIVING INTRAHYPOTHALAMIC IMPLANTATION OF IN MICROPELLETS WITH OR WITHOUT MER-25 AT 4 DAYS OF AGE AND A SUBCUTANEOUS INJECTION OF TERONE PROPIONATE (TP) AT 5 DAYS OF AGE.

Intrahypothalamic implants	Subcutaneous injection	Number of treated rats	S/F[a]	VO (day)	Onset of PVE (day)	IS[b] (%)
None	Oil	8	F(8)	41 ± 1	–	0
None	30 μg TP	5	S(5)	41 ± 2	74 ± 6	100
Sham	Oil	7	F(7)	40 ± 1	–	0
Sham	30 μg TP	12	S(9)	38 ± 1	71 ± 4	75
			F(3)	38	–	
MER-25	Oil	11	F(11)	40 ± 1	–	0
MER-25	30 μg TP	15	S(10)	31 ± 2**	59 ± 4*	67
			F(5)	37 ± 3	–	

sterile and F = fertile rats. Number of animals are indicated in parentheses.

$\frac{\text{No. of sterile rats}}{\text{No. of treated rats}}$ × 100%. *, ** Significantly earlier ($P < 0.05$, < 0.02, respectively) compared with

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d Welfare of Japan.

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rved.

The method of the intracranial implantation of paraffin pellets was a slight modification of the technique explained previously (12). The MER-25-paraffin mixture was tamped into stainless steel tubings (22 gauge, 0.4-mm i.d.) which contained a precut loading stylet. Weights of 11 control pellets were measured by an ultramicrobalance (Mettler ME 22) to the nearest 0.1 μg and the average weight (±SE) was 84.7 ± 1.8 μg. Since MER-25 was mixed with paraffin in 50% by weight, the amount of MER-25 per pellet averaged 42 μg. The animals were weaned at 22 days of age, and daily smears were recorded following the day of vaginal opening. To examine the ovarian compensatory hypertrophy (OCH), right ovaries were removed at 90 days of age, the remaining left ovaries 3 weeks later regardless of the day of sexual cycle. About 4 weeks after the last ovariectomy (around 120 days of age), the animals were sacrificed and the presence of intrahypothalamic micropellets was determined histologically. Ovaries were fixed in Bouin's solution, cut serially in paraffin, and stained with Delafield's hematoxylin and eosin. Those animals having polyfollicular ovaries without fresh corpora lutea (CL) were determined sterile. The significance of differences was tested with Student's *t* test.

Results. The injection of 30 μg of TP to 5-day-old female rats seemed a threshold amount of the steroid for induction of sterility, since only 9 out of 12 animals (75%) that received the sham-implantation at 4 days of age and a subcutaneous injection of TP at 5 days of age (group IV) became sterile by 90 days of age. On the other hand, TP injection of 5-day-old rats (group II) invariably induced sterility. Out of those 15 animals which were implanted with MER-25-paraffin mixture at 4 days of age and given a subcutaneous TP injection at 5 days of age (group VI) only 10 rats (67%) developed sterility; the remaining 5 had fresh CL in their ovaries (Table I). The micropellets of MER-25-paraffin mixture were found in suprachiasmatic nucleus (SC), anterior hypothalamic area (AHA), dorsomedial hypothalamic nucleus (DMH), or posterior hypothalamic nucleus (PH) of those rats which had received a TP injection neonatally and which had newly formed ovarian CL (Fig. 1A, shaded circles). Animals in which the micropellets were located in SC, paraventricular nucleus (PVH), DMH, or PH in those rats which were given a TP injection neonatally became sterile (Fig. 1A, open circles). In TP-injected rats which received paraffin pellets without MER-25, and which contained fresh ovarian CL at autopsy, the micropellets were located in DMH or PH. In rats which were sterile at autopsy the pellets were located in POA, AHA, VMH, DMH, or PH (Fig. 1B, shaded and open

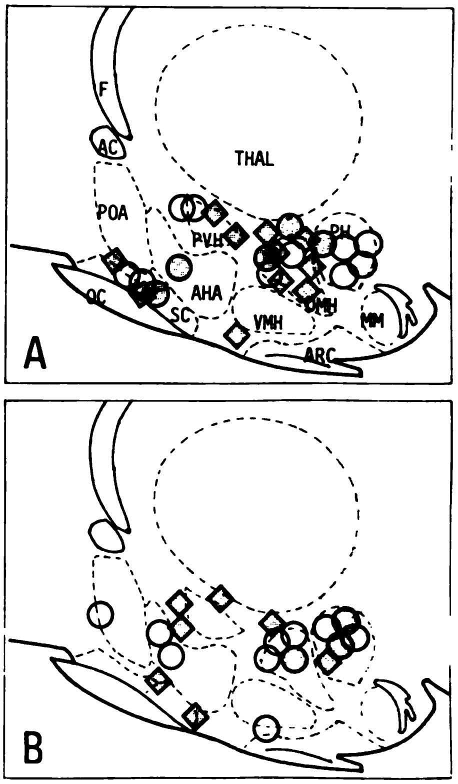

FIG. 1. Schematic parasagittal drawings of the hypothalamus showing loci of paraffin micropellets containing MER-25 + carbon marker (A) or marker only (B, sham operation). Those animals which had received a subcutaneous injection of 30 μg of TP at Day 5 became sterile (open circles) or fertile (shaded circles), while those which had been given an oil-vehicle injection became fertile invariably (shaded squares). Abbreviations: AC, anterior commissure; AHA, anterior hypothalamic area; ARC, arcuate nucleus; DMH, dorsomedial nucleus; F, fornix; MM, medial mammillary nucleus; OC, optic chiasm; POA, preoptic area; PH, posterior hypothalamus; PVH, paraventricular nucleus; SC, suprachiasmatic nucleus; THAL, thalamus; VMH, ventromedial nucleus.

TABLE II. MEANS (±SE) OF BODY WEIGHTS AT 90 DAYS OF AGE, OVARIAN WEIGHTS AND OVARIAN COMPENSATORY HYPERTROPHY (OCH) OF 3-WEEKS-INTERVAL IN THE SAME RATS AS IN TABLE I.

Group[a]	Number of rats	S/F[a]	Body weight (g)	Ovarian weight (mg)		OCH[b] (%)
				Right	Left	
I	8	F(8)	258 ± 10	38.7 ± 3.6	59.4 ± 4.3	56 ± 6
II	5	S(5)	233 ± 12	21.7 ± 2.1**	24.8 ± 1.7***	16 ± 5***
III	7	F(7)	236 ± 13	34.4 ± 2.3	54.0 ± 3.6	57 ± 6
IV	12	S(9)	260 ± 7	22.8 ± 1.8**	29.1 ± 1.5***	31 ± 7*
		F(3)	256	49.6	61.5	19
V	11	F(11)	255 ± 7	39.2 ± 2.6	60.9 ± 4.1	57 ± 6
VI	15	S(10)	253 ± 9	21.8 ± 2.0***	32.2 ± 3.6***	53 ± 13
		F(5)	262 ± 14	42.5 ± 7.5	43.6 ± 5.0	21 ± 29

[a] See Table I.

[b] $\text{OCH} = \dfrac{\text{Left ovarian weight} - \text{right ovarian weight}}{\text{Right ovarian weight}} \times 100\%$. *, **, and ***, Significantly smaller than spective controls (groups I, III, and V, $P < 0.02$, < 0.01, and < 0.001, respectively).

rcles, respectively). No significant correlaon seemed to be present between localizaon of intrahypothalamic micropellets and erility.

The days of vaginal opening (VO) and nset of persistent vaginal estrus (PVE) of ıose rats which had received MER-25 and 'P were significantly earlier than in those of ham-operated, TP-injected rats (Table I).)varian weights of the sterile rats from roups II, IV, and VI were also significantly maller than those of respective controls /hich had received oil injection (groups I, II, and V, respectively). OCH of sterile ats of groups II and IV was significantly maller than those of respective controls groups I and III, respectively; Table II).

Discussion. As stated in the previous reort, intrahypothalamic pellets with MER-5 (around 5 μg/pellet) implanted bilatrally in the 5-day-old females which immeiately received subcutaneous injection of 0 μg of TP failed to block sterilizing effect f the androgen (12). The ineffectiveness of ⁄IER-25in the previous experiment (12) ould be attributed to the fact that the mount of MER-25 was too small to block he overthreshold amount of TP (i.e., 50 ɩg) given immediately after operation. Iowever, it is shown in the present experiıent that a larger amount of MER-25 (42 ɩg/pellet on an average) also failed to block ıe sterilizing effect of a smaller amount of 'P (30 μg) which was given 24 hr after the ıtrahypothalamic operation. The fact that out of those 15 animals which had been iven MER-25 and TP (group VI) had fresh 'L in their ovaries as adults may be attributable not to the blocking influence of the antiestrogen on androgen-sterilization but to the threshold amount of TP applied to induce sterility, since 3 out of those 12 rats which had received sham-operation and TP-injection (group IV) also had fresh CL. It is concluded, therefore, that the interpretation of androgen sterilization as an exclusively estrogen function must be accepted with some reservation.

Summary. The influence of an estrogen antagonist, MER-25, on androgen sterilization was examined. Micropellets of MER-25-paraffin mixture, containing 42 μg of the antiestrogen per pellet on an average, were implanted bilaterally into the hypothalamus of 4-day-old female rats which received a subcutaneous injection of 30 μg of TP at 5 days of age. The amount of TP was, therefore, considered a threshold level for sterilization. In MER-25 implanted, TP-injected females, 10 out of 15 showed sterility, and the remaining 5 had fresh CL in their ovaries. No significant block by the antiestrogen of androgen-sterilization was detected. The previous interpretation of androgen-sterilization as an exclusively estrogen function must be, therefore, accepted with some reservation.

MER-25 was generously supplied by Merrell-National Laboratories, Cincinnati, Ohio.

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Kidney Response to Cold Stress and High Protein Intake[1, 2] (39404)

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stantial increases in kidney size have observed in animals exposed to cold or fed diets containing high levels of in (3-5). In fact, this effect of cold and ppears to be additive as evidenced by arkedly greater kidney enlargement in cold-exposed rats receiving a high in diet as compared to those fed a high hydrate diet (6). Whether achieved by r environmental temperature, the size se is the result of actual tissue h. Biochemically, such growth is evi- d by a normal tissue protein content vhile histologically, it is evidenced by plasia and hypertrophy of the glomer- bules, and interstitial cells (7, 8). Pre- ly, the cold-induced or diet-induced gement of the kidney represents a com- tory response to an added functional In the case of high protein diets, and bly in the case of cold exposure where loric intake can be increased twofold this added functional load may be d by an increased metabolic turnover rogen in the body. Indeed, when ani- re fed high protein diets, without cold ure, increases in the activities of renal es mediating the catabolism of amino e.g., glutamic dehydrogenase (4), and nitrogen, e.g., arginase (3), have reported. Such animals also exhibit an se in the rate of renal gluconeogenesis amino acid precursors (10). In con- the activities of renal glutamic-pyruvic and glutamic-oxalacetic transaminase are essentially normal in rats fed high protein diets (11). With cold exposure alone, slight increases in the activities of renal xanthine oxidase (12) and arginase (13) have been reported, as have increases in DNA content (1) and gluconeogenic capacity (9). As in the case of high protein diets, cold exposure alone does not affect renal glutamic-oxalacetic transaminase activity (1). These observations indicate that high protein intake or cold stress produce adaptive changes in several synthetic and degradative metabolic pathways of the kidney. Concomitant effects of cold stress and high protein intake on the activity of specific gluconeogenic and urea cycle enzymes associated with such pathways remain to be determined. Accordingly, the present study was directed at this problem.

Methods. Forty male Holtzman rats weighing from 280-300 g were used in this study. They were individually housed at 25° and fed a control, high carbohydrate diet for 10 days prior to experimentation. The diet contained, by weight, the following components: casein, 20%; sucrose, 73%; corn oil, 3%; mineral mix, 4%; and a complete vitamin supplement. Following this dietary adjustment period, the rats were divided into two treatment groups of 20 animals each. One group remained at 25° and the second group was placed in a cold room held at 5°. Ten animals in each group were continued on the control, high carbohydrate diet while the remaining animals were given a high protein diet. The latter contained by weight: casein, 85%; sucrose, 8%; plus the remaining ingredients at the levels described in the control diet. Food as well as water were available *ad libitum*. Food intake and change in body weight were recorded daily. Both the feeding and cold exposure periods were of 10 days' duration.

Thereafter, the animals were sacrificed,

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e opinions or assertions contained herein are the views of the authors and are not to be construed ial or as reflecting the views of the Department rmy or the Department of Defense.

onducting the research described in this report, stigators adhered to the "Guide for Laboratory Facilities and Care," as promulgated by the ttee on the Guide for Laboratory Animal Facili- Care of the Institutes of Laboratory Animal es, National Academy of Sciences-National h Council.

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the kidneys were removed, chilled in ice-cold saline, and weighed. One kidney from each animal was dried in a vacuum oven at 65° for 24 hr. The dried material was analyzed for total lipids with continuous (8 hr) ethyl ether extraction, and for total nitrogen by the micro-Kjeldahl procedure (14). The second kidney was used for preparation of tissue homogenates and for enzyme assays according to the following methods: glucose-6-phosphatase (GPase), the method of Cori and Cori (15); fructose-1,6-diphosphatase (FDPase), the method of Racker (16); phosphoenolpyruvate carboxykinase (PEPase), the method of Nordlie and Lardy (17) and Shrago and Lardy (18); glutamic-oxalacetic transaminase (GOT), the method of La Due *et al.* (19); glutamic-pyruvic transaminase (GPT), the method of Wroblewski and La Due (20); tryrosine alpha ketoglutaric transaminase (TGT), the method of Diamondstone (21); glutamic dehydrogenase (GDH), the method of Wergedal and Harper (4); arginase and arginine synthetase, the method of Brown and Cohen (22) and ornithine transcarbamylase, the method of Schimke (23). Since there were substantial differences in the final body weights and the size of the kidneys among the treatment groups, enzymatic activity was expressed as micromoles of product per minute per organ per 100 g body weight, as this method best expresses the total metabolic requirement of the kidney (5). All data were statistically evaluated with analyses of variance and significant *F* ratios were further evaluated with the Tukey multiple range test. Differences between means at the 0.05 level of probability were considered significant.

Results. As indicated in Table I, the cold-exposed animals lost body weight, the loss being significantly more pronounced in the group fed the high protein diet than in the group fed the control diet. In addition, the animals fed the high protein diet at 20° gained less weight than the corresponding control group. Cold-exposed animals consumed more food than the corresponding animals at 20°. However, food intake of the high protein group was lower than that of the control group at both 5 and 20°.

TABLE I. EFFECT OF COLD STRESS AND HIGH PROTEIN DIET ON SEVERAL KIDNEY COMPONENTS AND ENZYME ACTIVITIES TEMPERATURE AND DIET.

	20°		5°	
	Control	Protein	Control	Protein
Av change in BW/day, g	3.5 ± 0.28[a]	2.1 ± 0.19[c]	−3.3 ± 0.20[d]	−5.6 ± 0.3[cd]
Av food intake/day, g	17.6 ± 1.6	13.1 ± 1.9[c]	25.5 ± 1.3[d]	19.5 ± 1.7[cd]
Kidneys/pair, g	2.04 ± 0.02	2.46 ± 0.08[c]	2.23 ± 0.13[d]	2.94 ± 0.03[cd]
Moisture, %	77.71 ± 0.14	77.34 ± 0.12	77.81 ± 0.10	77.90 ± 0.15
Nitrogen, % wet wt	2.58 ± 0.10	2.52 ± 0.09	2.60 ± 0.08	2.53 ± 0.12
Lipids, % wet wt	2.93 ± 0.11	2.85 ± 0.12	2.89 ± 0.14	2.97 ± 0.22
Enzyme activity[b]				
Glucose-6-phosphatase	9.39 ± 0.69	19.26 ± 1.02[c]	9.72 ± 0.89	30.16 ± 1.83[cd]
Fructose-1,6-diphosphatase	2.47 ± 0.29	4.47 ± 0.36[c]	2.50 ± 0.18	7.50 ± 0.49[cd]
Phosphoenolpyruvate carboxykinase	1.72 ± 0.09	2.92 ± 0.30[c]	1.78 ± 0.12	4.51 ± 0.28[cd]
Glutamic-oxalacetic transaminase	18.77 ± 1.29	22.29 ± 1.43	19.89 ± 1.83	37.85 ± 2.19[cd]
Glutamic-pyruvic transaminase	0.49 ± 0.09	0.52 ± 0.03	0.48 ± 0.04	2.51 ± 0.12[cd]
Tyrosine-α-ketoglutaric transaminase	0.92 ± 0.21	0.88 ± 0.24	0.91 ± 0.19	0.96 ± 0.17
Glutamic dehydrogenase	15.16 ± 0.96	27.59 ± 1.38[c]	14.54 ± 1.13	38.89 ± 1.28[cd]
Arginine synthetase	0.14 ± 0.02	0.27 ± 0.03[c]	0.12 ± 0.03	0.31 ± 0.05[c]
Ornithine transcarbamylase	0.28 ± 0.05	0.65 ± 0.04[c]	0.25 ± 0.04	0.73 ± 0.08[c]
Arginase	9.43 ± 1.13	19.74 ± 1.83[c]	11.29 ± 0.93	20.75 ± 1.90[c]

[a] Mean ± SE from 10 rats.

[b] Micromoles/min/100 g BW ($P \leq 0.05$).

[c] Indicates significant ($P \leq 0.05$) difference from controls, same temperature.

[d] Indicates significant ($P \leq 0.05$) difference from 20°, same diet.

pared to the controls, high protein
caused an enlargement of the kid-
ne increase being significantly more
nced at 5° than at 20°. Neither diet
d stress had any effect on the mois-
oid, or nitrogen content of the kid-

oth environmental temperatures, the
es of GPase, FDPase, PEPase,
and of the three urea cycle enzymes
nhanced by the high protein diet.
rmore, the activities of GPase,
, PEPase, and GDH were signifi-
igher when the high protein diet was
° as compared to 20°. In addition,
protein diet stimulated the activity
T and GPT only when fed at 5°.
cold nor high protein intake af-
the activity of TGT. Cold had no
n enzymatic activity in animals fed
trol diet.

ssion. Initial stages of cold exposure
ciated with a stimulation of shiver-
mogenesis which may be largely de-
on glucose as an energy source.
this period, a marked enhancement
tic gluconeogenesis takes place, ap-
in response to an increased energy
of the cold environment (9). The
data demonstrate that, in addition
ver, the kidney is an effective gluco-
c organ in the cold environment
etary protein is the major source of
In many respects, gluconeogenic re-
of the kidney and the liver to the
otein intake are similar. The activi-
the urea cycle enzymes increase in
gans (24, 25) as do the activities of
FDPase, and GDH (4, 25). In con-
the liver, renal GOT, GPT, and
not respond to high protein diets
d in the warm environment (25).
r, as the present data indicate, the
of GOT and GPT is stimulated in
environment. These findings sug-
t aspartic acid, alanine, and tyrosine
significant precursors of renal glu-
he warm environment. This sugges-
upported by a report showing that
does not stimulate the rate of gluco-
sis in perfused kidney (26). The
data, however, also suggest that as-
id and alanine have significant roles
in the renal synthesis of glucose in animals subjected to cold stress and when limited to low carbohydrate intake. These findings would further imply that the enhanced activities of GOT and GPT observed under cold stress represent the maximal rate of gluconeogenic processes since the two enzymes were stimulated by dietary and environmental conditions which impose an apparent, maximal demand for glucose. In general, it appears that enhanced gluconeogenesis in cold-exposed animals fed high protein diets is substrate-induced, as a result of increased intake and catabolism of dietary protein, and not induced by cold per se.

Other factors which influence or control renal gluconeogenic capacity remain to be clarified. It would appear likely that increases in adrenocortical and thyroid activity observed during the initial stages of adaptation to cold stress (27) are responsible, at least in part, for enhancing the activities of renal gluconeogenic enzymes. Specific enzymes which respond to such hormonal stimuli include GPase (28), PEPase (29), GPT and GOT (30), and FDPase (31). A recent report indicating an increase in the renal GOT only when high protein intake was combined with the administration of hydrocortisone (25) gives credence to such a conclusion.

The present data demonstrate, as have the data of many others (32–34), that cold-exposed animals increase their food intake to compensate for heat loss to the environment. However, animals fed the high protein diet ate less than those fed the control diet; consequently, the loss in body weight was more severe. Apparently, cold stress failed to overcome the hypophagia associated with the intake of high protein diets. This observation confirms and extends the conclusion of Beaton (34) who reported that rats exposed to cold for 8 days and fed a 40% casein diet ate less than rats receiving a diet containing 5 or 20% casein.

Metabolic events related to adaptation to high protein diets are not thoroughly understood; however, prominent among these are depression of food intake and body weight gain, hypertrophy of the kidney and the liver, alterations in the activity of several enzyme systems participating in the catabolism of amino acids, and changes in the profile of plasma-free amino acids (5). In general, such metabolic events seem to be related to the animal's capacity to dispose of a large influx of amino acids resulting from ingestion of excess protein. If the influx exceeds the oxidative capacity for specific amino acids, the tissues may be presented with an imbalanced amino acid mixture which would adversely affect the food intake and the synthesis of new proteins. In this respect, previous data from several laboratories show that cold-exposed rats readily consume diets having a moderately imbalanced pattern of amino acids (35–37). However, when the imbalance was made more severe food intake was depressed to the level observed in a warm environment (38). Such data also show that cold-exposed animals oxidize the excess amino acids more rapidly than normothermic rats at 20° (39). The foregoing observations suggest that rats maintained on a high protein diet do not have the capacity to degrade excess amino acids at a rate that would have allowed an increase in food intake commensurate with the elevated energy demands of a cold environment.

Summary. Compared to rats fed a high sugar diet, consumption of a high protein diet caused an enlargement of the kidneys, the increase being more pronounced at 5° than at 20°. High protein intake enhanced activities of renal glucose-6-phosphatase, fructose-1,6-diphosphatase, phosphoenolpyruvate carboxykinase, glutamic dehydrogenase, and of the urea cycle enzymes. However, the activities of the gluconeogenic enzymes were higher at 5° than at 20°. In contrast, enzymatic activity was not changed in cold-exposed animals fed the high sugar diet. Since cold exposure stimulates food intake, it appears that enhanced renal gluconeogenesis observed in this study is substrate-induced rather than induced by cold per se.

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Reduced Blood and Marrow Neutrophils and Granulocytic Colony-Forming Cells in Sl/Sl^d Mice[1] (39405)

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The Sl/Sl^d mouse is anemic (1), has reduced numbers of megakaryocytes but normal levels of blood platelets (2), and its defective hematopoiesis is thought to be due to a defect in the hematopoietic microenvironment. This latter conclusion is based on a series of observations. The animal will not support growth of normal sized spleen colonies on its own spleen (3), but if intact littermate spleens are transplanted in Sl/Sl^d they will support normal colony growth (4). Transplantation of intact littermate spleens improves the anemia (5) but intravenous injection of spleen or marrow cells does not (3). Marrow or spleen cells from Sl/Sl^d grow normally when injected into irradiated littermates or syngeneic W/W^v (3). These studies thus suggest that its hematopoietic stem cells are normal, that levels of "poietins" are adequate and by default implicate the poorly defined microenvironment. The reduction of megakaryocytes (1) indicates the defect is not confined to erythropoiesis and suggests that feed-out from the pluripotent stem cell compartment might be reduced. If so, then reduced neutrophils and neutrophil precursors should also be present. In this study we measured blood and marrow neutrophils and neutrophil precursors, number of marrow cells forming granulocytic colonies in semisolid culture, marrow growth in liquid culture, and production of colony-stimulating activity from macrophages.

Materials and methods. All methods used have been reported in detail and will only be summarized.

Mice were purchased from Jackson Laboratories, Bar Harbor, Maine. Mice of various ages were used, but in each experiment Sl/Sl^d and the normal littermates (+/+) were matched for age. Blood was obtained by puncturing the lateral orbital sinus with a microhematocrit tube containing heparin. Duplicate total leukocyte counts were done electronically and 200 cell differential counts were done on Wright's stained smears (6). Nucleated cells were washed from both humeri, duplicate counts were done electronically, and results were expressed as the mean value from both humeri (6). The method removes more than 90% of nucleated cells and is reasonably precise in that counts rarely vary by more than 10% between the humeri of the same mouse. An aliquot of the marrow suspension is passed through a millipore filter (pore size 45 μm), cells on the filter stained for peroxidase and the percentage peroxidase positive cells determined by counting 500 cells.

For semisolid culture, 10^5 nucleated cells from a femur are added to 1 ml of methyl cellulose, CMRL culture media, horse serum, and colony-stimulating activity from L cells, and the mixture was added to a petri dish (7). Dishes are incubated at 37° in humidified 10% CO_2 and colonies (more than 50 cells) counted after 4 and 7 days. Liquid cultures in test tubes, 12 × 75-mm polyethylene (Falcon plastics), were set up in the same media but without methyl cellulose (8). One culture tube from each mouse was sacrificed at 2, 4, 6, and 8 days of culture and total cells were determined electronically.

Results. Twelve-week-old Sl/Sl^d mice were compared with littermate controls (+/+) with respect to blood and marrow (Table I). Moderate anemia was present in Sl/Sl^d with hematocrit values averaging 65% of those of the +/+. Total leukocyte count on the first tube of blood drawn from the orbital sinus did not differ between the two groups. However, the percentage of neutro-

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TABLE I. GRANULOCYTOPOIESIS IN Sl/Sl^d MICE AND THEIR LITTERMATE CONTROLS

	Mice*		
	Sl/Sl^d (Mean ± SE)	+/+	*P* values
veight (g)	21 ± 1	22 ± 1	NS
itocrit (%)	31 ± 1	48 ± 1	<0.01
ocytes (thousands/mm^3)	4.1 ± 0.2	4.3 ± 0.2	NS
ophils (thousands/mm^3)	0.8 ± 0.1	1.4 ± 0.2	<0.05
(humerus)			
nucleated cells (millions)	5.7 ± 0.4	9.6 ± 0.4	<0.01
idase positive (%)	19 ± 2	35 ± 4	<0.05
peroxidase positive	1.2 ± 0.1	3.5 ± 0.5	<0.01
iies/10^5 nucleated cells	105 ± 8	114 ± 12	NS
colony-forming cells/humerus (millions)	0.06 ± 0.01	0.12 ± 0.02	<0.01

e mice per group. ** From Student's *t* test; NS indicates nonsignificant or *P* of greater than 0.05.

vas only 19% in Sl/Sl^d as compared to
n +/+, so the total neutrophil concen-
ı in Sl/Sl^d was reduced to 57% of that
+ (Table I). The total number of lym-
tes per cubic millimeter was slightly
in Sl/Sl^d than in +/+, but the differ-
vas not statistically significant. Leuko-
ounts and neutrophil concentration
ılso determined on the seventh tube of
drawn from the orbital sinus. Total
were significantly less in Sl/Sl^d than
+ (1900 ± 100 vs 2600 ± 200) and
was an even greater reduction in neu-
ls than in the first tube (Sl/Sl^d reduced
% of +/+, as compared to 57% in first
ounts).

al nucleated cells per humerus were
d to 59% of control in Sl/Sl^d (Table
e percentage of peroxidase positive
which correlates well with the percent-
neutrophils and neutrophil precursors
d in Wright's stained smears (6), was
educed to 54% of controls. Conse-
y, a profound reduction (34% of con-
as present in total peroxidase positive
er humerus (Table I). The proportion
rrow cells forming colonies in semi-
nedia was quite similar in Sl/Sl^d and
However, as a result of the decrease
l cells per humerus, the total number
ony-forming cells was decreased sig-
ıtly in Sl/Sl^d (Table I).

studies were repeated in 8-week-old
and all of the above differences be-
Sl/Sl^d and littermates were confirmed
s not shown).

ony size was estimated on Day 4 and Day 7 with the observer being unaware of whether the plate contained marrow from Sl/Sl^d or from +/+. Colonies were estimated as containing 50–100, 100–500, 500–1000, or greater than 1000 cells. Clusters (20–50 cells) were also scored. No significant difference in colony size was discerned between Sl/Sl^d and +/+. For example, 32% of all colonies and clusters were estimated to contain more than 100 cells in Sl/Sl^d as compared to 31% in +/+.

In order to further study growth rate of cells, which colony size presumably reflects, studies were done in liquid culture so that accurate cell counts could be done. One-hundred-fifty-thousand nucleated marrow cells from each of 4 Sl/Sl^d and +/+ were cultured. At 2, 4, and 6 days nucleated cells were 148,000, 182,000, and 235,000 in +/+ and 152,000, 180,000, and 232,000 in Sl/Sl^d cultures. In a repeat study, carried to 8 days, again no significant difference was observed between the number of cells in cultures from +/+ or Sl/Sl^d.

Colonies were removed from plates with a Pasteur pipet and smeared on glass coverslips and stained. Smears were also made from liquid cultures. At 4 days most cells appeared to be neutrophil precursors and by 7–8 days almost all appeared to be macrophages. There was no evident difference in morphologic appearance of cells between cultures of Sl/Sl^d and +/+ marrow.

Peritoneal macrophages of Sl/Sl^d were as efficient as those from +/+ when used as a source of colony-stimulating activity (Table II). Addition of peritoneal cells in excess o[illegible] the number needed to produce maximal colony stimulation failed to yield any suggestion of production of an inhibitor by the Sl/Sl^d (Table II). The marrow used in the second experiment shown in Table II was from 32-week-old mice and unlike the previously noted studies with 8- and 12-week-old animals there was a significant ($P < 0.01$) reduction in the concentration of colony forming cells in Sl/Sl^d marrow whether stimulated with +/+ or Sl/Sl^d feeder layers or with conditioned media from L cells.

Discussion. Reduced numbers of neutrophils are present in blood and marrow of Sl/Sl^d as compared to +/+ littermate controls. This is not unexpected since, in addition to anemia, the number of megakaryocytes is reduced in this mouse (2). Thus, three major cell lines are affected by the defect. This suggests that the primary problem may reside at the level of the pluripotent stem cell. If so, the defect could reflect a reduced stem cell compartment size (reduced rate of self-replication) or defective induction of differentiation from the compartment. As discussed in the introduction, the Sl/Sl^d defect appears to reside in the hematopoietic microenvironment rather than being an intrinsic defect of the stem cell. The W/W^v mouse, which does have defective stem cells (10), also has reduced marrow neutrophils (11) and megakaryocytes (11, 12) in addition to being anemic.

The hematopoietic microenvironment is poorly understood (13). It is not clear whether the marrow (and to a lesser degree the spleen) of mice contains some "positive" factor necessary for hematopoietic growth which is missing in other organs, whether the physical structure of the marrow is the key factor or whether other organs contain a "negative" environment.

Hormonal deficit(s) probably cannot be implicated since apparently normal hematopoiesis takes place in +/+ marrow or spleen implanted in Sl/Sl^d (5, 14). Erythropoietin levels appear to be at least appropriate to the degree of anemia (15). Production of colony-stimulating activity from Sl/Sl^d peritoneal cells was normal in our study as it was from bone marrow cells in preliminary reports from other laboratories (16, 17).

The cell-forming colonies of neutrophils and monocytes in semisolid culture is thought to be a more differentiated cell than the pluripotent stem cell. Whether it is a true stem cell, i.e., capable of extensive self-replication, remains to be proven unequivocally. This cell is often referred to as a CFU$_C$ (colony-forming unit in culture) while the pluripotent cell is termed a CFU$_S$ (colony-forming unit in spleen of irradiated

TABLE II. ABILITY OF MACROPHAGES FROM +/+ AND Sl/Sl^d TO STIMULATE COLONY FORMATION FROM +/+ AND Sl/Sl^d MARROW.

Experiment number	Source of colony-stimulating activity		Target marrow (10^5 marrow cells)	
			+/+	Sl/Sl^d
			Number colonies/plate (Mean ± SE)	
	Conditioned media from +/+*		39 ± 4	–
	Conditioned media from Sl/Sl^d		32 ± 3	–
2	Feeder layer of peritoneal cells from +/+**	(5×10^5 cells)	95 ± 4	68 ± 3
	Feeder layer of peritoneal cells from Sl/Sl^d	(5×10^5 cells)	92 ± 6	72 ± 6
	Feeder layer of peritoneal cells from +/+	(10^6 cells)	96 ± 7	71 ± 2
	Feeder layer of peritoneal cells from Sl/Sl^d	(10^6 cells)	91 ± 5	67 ± 10
	L-Cell-conditioned media***		104 ± 4	69 ± 2

* Peritoneal macrophages were collected by placing a 1-cm^2 of cellulose acetate in the peritoneum for 1 week. Such membranes are covered with a uniform monolayer (9). Macrophage monolayers were incubated for 1 week in CMRL tissue culture media and the supernate harvested and used as a CSA source. Four membranes from four mice in each group were studied.

** The peritoneal cavity of freshly killed, untreated mice was lavaged with CMRL media. Approximately 60% of the harvested cells appeared to be macrophages and the remainder appeared lymphoid. Peritoneal cells from three mice in each group were studied.

*** Used at maximal stimulation level.

The best evidence that the CFU$_C$ from the CFU$_S$ is found in studies of W^v mouse. CFU$_S$ is clearly defective, W^v marrow contains a normal con- ion of CFU$_C$ (18). The total number U$_C$ is reduced because of the total of nucleated cells is reduced (10), y grow colonies of normal size *in* 8; and unpublished studies from our ory).

concentration of CFU$_C$ in marrow rmal in Sl/Sl^d aged 8 and 12 weeks, ificantly less than that of controls in oup of 32-week-old mice. However, e total number of nucleated cells in was reduced in the Sl/Sl^d, total er humerus were reduced in all age studied, in basic agreement with pre- y reports from other laboratories). McCulloch *et al.* (18) found the ration of CFU$_C$ in spleens of Sl/Sl^d duced in three of four studies. Suth- Till, and McCulloch (19) studied the repopulation of the marrow and of irradiated Sl/Sl^d recipients of +/+ as compared to +/+ recipients. The repopulation of CFU$_C$ was sugges- educed and peroxidase-positive cells ly reduced in Sl/Sl^d marrow and both re even more profoundly reduced in The reduction in CFU$_C$ certainly is proportionate to the overall reduc- neutrophils and is compatible with ct primarily affecting rate of feedout from the CFU$_S$ compartment. The ration of CFU$_S$ in Sl/Sl^d marrow is (3, 22) as it is in the spleen (21). hy found that the total number of femur, spleen, and blood was also (22). CFU$_S$ also are decreased in er of Sl/Sl^d (23). Therefore, reduced from the compartment could simply xpression of reduced compartment alternatively, there could be a defect of initiating differentiation.

ormal growth of cells in semisolid or ltures is what would be anticipated of apparently normal growth of Sl/ in irradiated normal littermates (3,

ugh megakaryocytes are reduced in , platelet counts are normal in Sl/Sl^d W/W^v (12). The normal platelet count probably reflects increased platelet production per megakaryocyte (2, 11, 24). The W/W^v usually maintains normal blood neutrophil levels despite a 50% reduction in marrow neutrophils and neutrophil precursors (11). Since the Sl/Sl^d had a more severe reduction in marrow neutrophils than the W/W^v, it is not surprising that they were neutropenic. Sl/Sl^d also are more anemic and more radiosensitive than W/W^v (5), so it seems probable the stem cell compartment is more severely compromized by the Sl/Sl^d defect.

As judged by response to epinephrine, patients with neutropenia have a greater proportion of cells in the marginal blood pool than in the freely circulating pool (25). The mouse has no change in leukocytes after epinephrine injection (unpublished observations), but an assessment of the size of marginal and circulating pools can be obtained from the ratio of neutrophil counts in the first and seventh tube of blood obtained from the orbital sinus (6). The first tube of blood from the orbital sinus as well as the first drop from a tail vein or the first sample from the vena cava have significantly higher leukocyte concentrations than does the aorta of the mouse (6). As repeated tubes are drawn from the orbital sinus, leukocyte concentration declines and by the seventh tube approximates counts from the aorta. The most reasonable explanation seems to be that the marginal as well as the circulating pool is represented in the first tube while the aorta represents only the circulating pool. Thus, the ratio of first to seventh tube counts provides an estimate of the relative size of the marginal and circulating pools. In +/+ this ratio was 60% while in Sl/Sl^d it was 45%. This suggests that the circulating pool was reduced to a greater degree than the marginal pool in Sl/Sl^d animals.

Summary. The Sl/Sl^d mouse has been shown to have anemia and reduced megakaryocytes, but neutrophil levels in blood and marrow have not been studied previously to our knowledge. It proved to be neutropenic with reduced neutrophils and neutrophil precursors in marrow. The concentration of marrow cells forming granulocytic colonies in semisolid media was normal in young mice, but the total number per

humerus was reduced. *In vitro* growth appeared normal in both semisolid and liquid culture, and peritoneal cells produced colony-stimulating activity normally. These results are compatible with previous studies indicating the Sl/Sl^d defect lies in the hematopoietic microenvironment rather than being a primary cellular defect, and suggest feedout from the pluripotent stem cell compartment may be reduced.

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nship between Age and Thymic Function in the Development of Leukemia in AKR Mice (39406)

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mice develop spontaneously lym- leukemia and have served as or study of murine leukemia virus lthough the virus is present in the efore birth, disease is not manifest mice are at least 6 months old (4, nning at that age, frank lymphoid s develop in most parenchymal or- ten with a thymoma and marked ytosis (1-5).

mmunopathologic mechanism re- for this latent period is largely but several key observations have scribed. Thymectomy before 8 f life markedly reduced the inci- leukemia (1, 6). Leukemogenesis ccelerated in these thymectomized with the typical latency period, by tation of young thymic grafts (6). er, young thymectomized mice ted with a 6-7 month syngeneic raft develop leukemia without a la- riod. In addition, the incidence of is reduced in AKR-C3H chimeras se observations have led investiga- opose that the latency period is due esence of thymic regulatory or sup- ell activity in young mice (7, 8).

herein investigated the role of the and its relation to the age of the the development of leukemia in ce. In addition, we have tested the is that thymic derived cells, capa- hibiting neoplastic transformation ression, are present in young mice ost with age by repetitive transplan- either young (1 month) or old (6 hymic grafts.

als and methods. Mice. AKR/J mice were obtained from Jackson Laboratories, Bar Harbor, Maine. Mice were housed 10 per cage and fed standard lab chow and water *ad libitum*. Other strains of mice used in this experiment: A/LN, BALB/C, NZB, and DBA/2 were obtained from the Rodent and Rabbit Production Unit, National Institutes of Health, Bethesda, Md. Only male mice were used in this study.

Survival studies. Two-month-old AKR mice were randomly divided into two groups. One (consisting of 25 mice) was grafted with a 1-month-old AKR thymus every fortnight. The second (20 mice) was untreated and served as a control group.

Additional 2-month-old AKR mice were divided into three groups: (i) Thirty mice were thymectomized and 10 days later given a 6-month AKR thymus graft. (ii) Thirty mice were sham-thymectomized and 10 days later given a 6-month AKR thymus graft. (iii) Twenty-five mice were sham-thymectomized and not grafted.

Sixty-nine 6-month-old AKR mice were ramdomized into three groups of equal size. The first group was untreated. The second received repetitive transplants every fortnight of 1-month-old AKR thymic grafts. A third group received every fortnight thymic grafts from 6-month-old AKR mice.

Thymic transplants. Donor mice were sacrificed by cervical dislocation and thymectomy was performed. The thymus from a 1-month animal was divided into two lobes so that each recipient received one lobe. The thymus from 6-month animals was used intact. In all cases the contiguous lymph nodes and fatty tissue were carefully dissected from thymic graft and was implanted subcutaneously in the axilla of recipient mice; the wound was closed with a stainless steel clip. Mice receiving repetitive transplants were grafted initially in the axilla and thence sub-

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ed.

sequently in the flanks and dorsum. The survival of the transplants were confirmed by palpation of the graft during the survival phase and by histologic examination when moribund. Additionally, the survival of the transplants was confirmed, in mice sacrificed, for mitogen studies, by light microscopy with further identification of thymocytes using anti-theta sera (9). All mice were followed for survival and autopsied when moribund. Survival data between groups was prepared using the Kolmogorov-Smirnov nonparametric test (10).

Transplantation of AKR thymomas. Spontaneously occurring thymomas were removed at autopsy (performed on moribund animals) and gently minced into tissue fragments 2 × 2 mm in Hanks Balanced Salt Solution (Grand Island Biologic Co., Grand Island, N.Y.). The fragments were placed subcutaneously in the flank of groups of eight AKR mice of each of the following ages: 4, 8, 12, 20, 28, and 36 weeks. Additional recipients were eight 8-week-old AKR mice thymectomized 2 weeks earlier. The size of the graft and survival of recipients was followed daily. Similar grafting experiments were performed using 4- and 24-week NZB, BALB/c, DBA/2, and A/LN mice.

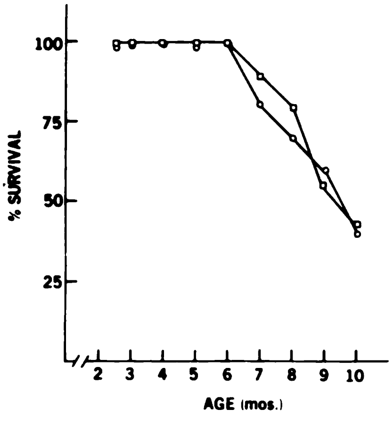


FIG. 1. Percentage survival plotted against age in untreated control mice (□—□) and mice transplanted every fortnight with 4-week AKR thymus (O—O) beginning at age 8 weeks.

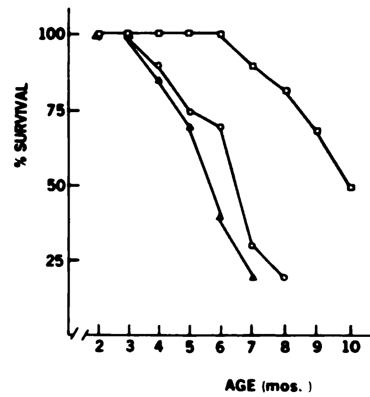
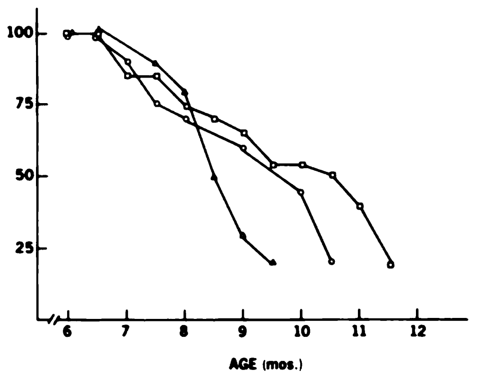


FIG. 2. Percentage survival plotted against age in (a) mice undergoing sham thymectomy at 8 weeks of age (□—□); (b) mice thymectomized at 8 weeks of age transplanted 10 days later with a 6-month thymic graft (Δ—Δ); and (c) 8-week-old mice undergoing a sham thymectomy and transplanted 10 days later with a 6-month thymic graft (O—O).

Mitogen studies. Groups (four mice per group) of 6-month and 10-month AKR mice repetitively transplanted from 8 weeks of age with thymic grafts from 4-week-old AKR mice were sacrificed by cervical dislocation. Groups (four to eight mice per group) of 2-, 6-, and 10-month-old untreated AKR mice served as controls. The spleen, mesenteric, and femoral lymph nodes were removed and placed in RPMI 1640 media (Grand Island Biologic Co.). Single-cell suspensions of spleen and lymph nodes were prepared by passing the tissue through sterile 22-gauge nylon mesh, teasing the resultant fragments and shearing clumps through a 25-gauge needle. Viability was confirmed by trypan blue exclusion. Each animal was studied individually. The cell suspensions were cultured in microtiter plates as previously described (11). To sextuplicate cultures, from each animal, were added 100 μl of each mitogen: phytohemagglutinin-P (PHA-P), 0.1% final concentration (Difco Labs, Detroit, Mich.); Concanavalin A (Con A), 0.25 μg/culture (Calbiochem, San Diego, Calif.); *Escherichia coli* lipopolysaccharide (LPS), 50 μg/culture (Difco Labs); and polyriboinosinic acid, polyribocytidylic acid [poly(I)·poly(C)] 50 μg/culture (P-L Biochemicals, Milwaukee, Wis.); or RPMI 1640 alone. Cultures were incubated for 72 hr at 37° in a 5% carbon dioxide, 80% oxygen, 15% nitrogen-humidified atmosphere. Eighteen hours before harvest, each received 1 μCi of [methyl-^{3}H]thymidine ([^{3}H]Tdr) (1.9 Ci/mmole, Schwartz-Mann, Orangeburg, N.Y.) in 20 μl of media. Cultures were harvested with



'IG. 3. Percentage survival plotted against age in 6-month, unmanipulated, AKR mice (□—□); (b) onth mice grafted every fortnight with 4-week syn- :ic thymus (○—○); (c) 6-month mice grafted every ıight with 6-month thymus (△—△).

automated multiple sample harvester.

!esults. There was no evidence to suggest inherent resistance of young AKR mice /oung AKR thymic cells to development leukemia. AKR mice, 8 weeks old at ly entrance, repetitively grafted with 4- :k grafts had identical survival to un- ıted controls (Fig. 1). Mortality was ac- :rated by grafting 8-week-old AKR mice h 6-month syngenic thymic grafts (Fig. However, the prevalance of leukemia ; similar in the 8-week-old AKR mice :iving a single 6-month graft, whether or they had been thymectomized (Fig. 2).)etitive grafting of 4-week thymic tissue) mice 6 months of age at study entrance not prolong survival (Fig. 3). However, e 6 months old at study entrance, receiv- repetitive grafting of 6-month thymic ue, developed leukemia earlier (Fig. 3). : 50% mortality for this group was signif- ıtly shorter ($P < 0.01$) than the control up.

he AKR (thy 1.1) thymus transplants /ived in the recipients. This was con- ıed by palpation, histology, and use of -thy 1.1 sera. The growth rate and sur- ıl of transplanted thymomas in AKR e was unrelated to age (Table I). As ht be expected, AKR thymomas do not 'ive in θ-C3H (thy 1.2) strains of mice :B, BALB/c, DBA/2, or A/LN). It is :worthy that no growth was observed in .N mice as they, unlike the other strains, : the theta-AKR (thy 1.1) allele, and thy 1.1 has been observed on AKR lymphoma cells (12).

There was progressive loss of PHA-P and Con-A responsiveness of spleen cells as AKR mice aged (Fig. 4). A lesser relation in responses to pokeweed mitogen and LPS (Primarily B cell mitogens) was observed with age (Fig. 4). Lymph node cells which respond minimally to T cell mitogens also showed a decrease with age (Fig. 5).

Discussion. It is generally accepted that thymectomy of young AKR mice markedly reduces the incidence of leukemia. However, several critical findings have suggested that, in addition to harboring the murine leukemia virus, the AKR thymus may play a role in the long latency period before disease becomes manifest (7, 8). Treatment of AKR mice with antilymphocyte serum accelerated leukemogenesis (13). Further, leukemia is retarded in allogeneic bone marrow chimeras as well as in tetraparental ovum fusion-derived AKR chimeras (7, 8, 14, 15).

The present study strongly indicates little or no significant active role for the AKR thymus in retarding leukemogenesis. Subcutaneous thymoma grafts of AKR origin grew equally well in AKR recipients of all ages. Further, repetitive transplantation of young syngeneic thymic tissue into 8-week or 6-month mice, did not alter survival. In

TABLE I. RESPONSE TO SUBCUTANEOUS TRANSPLANT OF AKR THYMOMA.[a]

Recipients	Age[b]	Growth[c]	Survival[d]
AKR/J	4	15.9	39.4
AKR/J[e]	8	15.3	35.7
AKR/J	8	13.8	41.2
AKR/J	12	16.7	36.8
AKR/J	20	15.1	44.2
AKR/J	28	17.4	32.1
AKR/J	36	14.9	27.4
NZB	4	0	>90
NZB	24	0	>90
BALB/c	4	0	>90
BALB/c	24	0	>90
DBA/2	4	0	>90
DBA/2	24	0	>90
A/LN	4	0	>90
A/LN	24	0	>90

[a] Groups of four to eight mice.

[b] Age in weeks.

[c] Mean time in days to reach palpable tumor mass of $1 \times 1\ \text{cm}^3$.

[d] Mean survival in days.

[e] Thymectomized at age 6-weeks.

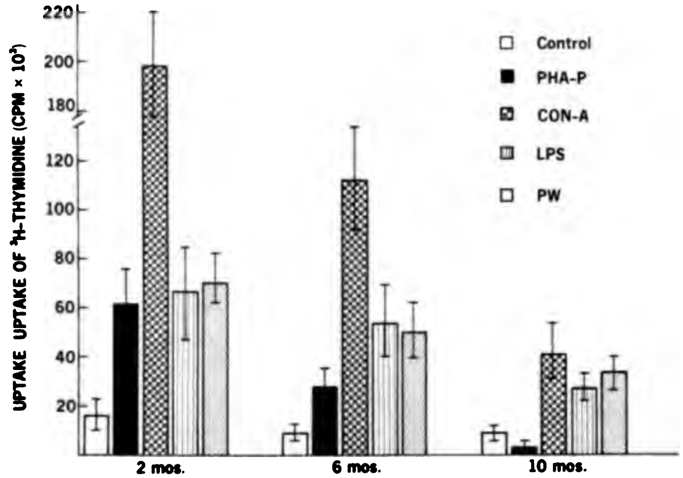


FIG. 4. Incorporation of [^{3}H]thymidine by spleen cells from AKR mice 2, 6, or 10 months of age.

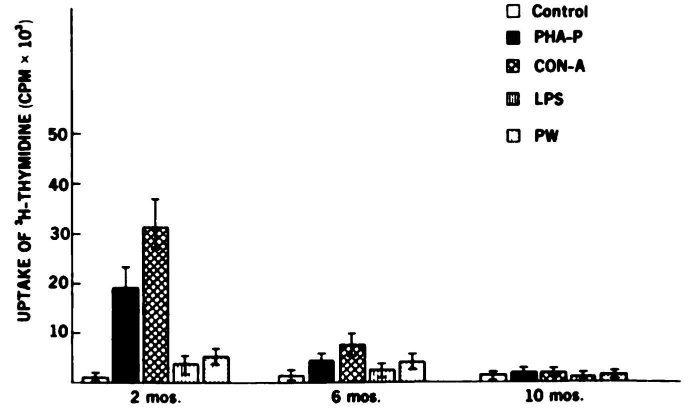


FIG. 5. Incorporation of [^{3}H]thymidine by lymph node cells from AKR mice 2, 6, or 10 months of age.

fact, repetitive grafting of 6-month thymus into mice, beginning at age 6 months, accelerated disease. This acceleration of leukemogenesis may be secondary to the larger potential source of premalignant (thymus) tissue. These results are consistent with observations that spontaneous antilymphoma cytotoxic reactions of preleukemic AKR mice are not T-cell dependent (16).

Immunotherapy of leukemia in AKR mice has been largely disappointing. Although antibody to virus exists it occurs primarily as antigen-antibody complex (17, 18). Cell mediated immunity to virus has not been demonstrated. Interferon administration retards leukemogenesis (19). Similarly, pyran-2-succinic anhydride-4,5-dicarboxytetrahydro-6-methyl-anhydride polymer (NSC-46015), an interferon inducer, retards disease (13). Finally, lethal irradiation of leukemic AKR mice, followed by restoration with bone marrow cells from germ-free DBA mice prevents leukemia (14). The present study militates against use of syngeneic tissue transplants and in favor of further understanding the host-viral relationship before immunotherapeutic manipulation.

The mitogen studies indicate a progressive loss of response to the T cell stimulators Con-A and PHA-P. This is similar to studies in patients with chronic lymphocytic leukemia and may reflect either a loss of T cells or a dilution of T cells by either a null cell or B cell population (20–22). It is of particular interest that a decrease in responsiveness of pleen and lymph node cells to Con-A and HA-P preceded frank leukemia. Further, nice receiving thymic grafts had identical nitogen responsiveness as unmanipulated ontrols. Neoplastic transformation appears o occur, therefore, more as a consequence f the virus natural history than of the imnunologic condition of the AKR host.

Summary. The characteristic latency peiod of 6 months was noted for the developnent of leukemia of AKR mice. Growth of ransplanted syngeneic thymomas occurred qually well in 4-week- as 28-week-old reipient AKR mice. Repetitive grafting of hymic tissue from 4-week donors into AKR nice 8 weeks or 6 months old at initiation of tudy failed to retard leukemogenesis. This esult is contrary to the hypothesis that the atent period for leukemia in AKR mice is roduced by thymic suppressor cells in oung AKR mice. Further, transplantation f 6-month thymic tissue into either 8-week r 6-month recipients accelerated disease. here was a progressive loss of responsiveess to phytohemagglutinin and concanaalin A in AKR mice with age. The responiveness to mitogens during frank leukemia vas similar to that described in patients with hronic lymphocytic leukemia.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 408–410 (1976)

Bacterial Synthesis of Substance Similar to Human Chorionic Gonadotrophin (39407)

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Recently, Livingston and co-workers (1) described the isolation of an organism from urine of cancer patients which, when cultured *in vitro*, produced a substance immunologically related to human chorionic gonadotropin (HCG). A sample of this material obtained from Dr. Livingston and examined immunologically for the presence of HCG using the hemagglutination inhibition technique (2) indeed confirmed the observations reported by them and persuaded us to proceed further.

We requested and received cultures of the organism from Dr. Livingston, and we ourselves were able to isolate an organism, which secreted HCG-like material, from the urine of a terminal colon carcinoma patient (A. K.).

The following report presents our data confirming the provocative findings of this group.

Material and methods. Culture of the organisms received from Dr. Livingston, as well as that isolated from the urine of A. K., a patient terminal with colon carcinoma, followed the procedure of Livingston *et al.* (1). Harvesting of the fermentation was also according to these workers. Although the organism has been named *Progenitor cryptocides* by the Livingston group no such classification is described in "Bergey's Manual." We have made no attempt to further classify this organism.[1]

Solids obtained by acetone precipitation of the contents of the fermentation flasks were extracted with 0.1 *M* EDTA (pH 6.8) and centrifuged. The extracts were subjected to immunological assay for HCG by the following procedures: hemagglutination inhibition (2); radioimmunoassay (RIA) as described by Saxena *et al.* (3) using purified HCG labeled with I^{125} and an antibody raised to HCG beta-subunit and absorbed with the alpha-subunit of HCG in order to increase specificity.

The extracts were also examined for the presence of biologically active HCG by radioreceptor assay (RRA) (4) using highly purified HCG labeled with I^{125} and bovine corpora lutea membranes isolated by the procedure described by Saxena and co-workers (4). The effect of the bacterial "HCG" on mouse Leydig cell preparations was established through the cooperation of Dr. K. Sundaram at Rockefeller University (5).

One bacterial extract was further purified by chromatography on Concavalin A-sepharose as described by Dufau *et al.* (6).

To compare the effect of proteolytic enzyme on bacterial "HCG" and the human hormone, samples of each were incubated with trypsin (20 μg trypsin per I.U. HCG activity) for 6 hr at 37°, pH 7.5 in 0.1 *M* phosphate buffer. Residual hormone activity was determined by RIA as described above.

Results and discussion. Figure 1 presents radioimmunoassay data comparing highly purified HCG (NIH reference HCG) with the bacterial "HCG-like substance" elaborated in trypticase soy broth by cultures of the microorganism furnished by Dr. Livingston. Parallelism between the dose response curves for this isolate and pure HCG indicates a strong immunological relationship between the mammalian hormone and the bacterial substance, particularly since the antibody used was raised by immunization with the beta-subunit of HCG. Extracts of trypticase soy broth cultures of *Escherichia coli* and *Staphylococcus aureus* did not contain a similar substance. Additional evidence for the similarity between HCG and

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[1] Preliminary attempts at identification of the organisms secreting the HCG-like material indicated that the culture obtained from patient A. K. was a gram-negative rod. Confirmation by fermentation criteria indicated the organism was *Escherichia coli*. The two cultures obtained from Dr. Livingston were both gram-positive cocci.

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the bacterial product is found in the parallelism of the slopes of the curves obtained in the radioreceptor assay for purified HCG and the bacterial substance isolated from cultures of the organism obtained from Dr. Livingston, as well as the organism isolated in this laboratory from the urine of a terminal carcinoma patient (Fig. 2). Incubation of isolated mouse Leydig cells with the bacterial extracts stimulated production of testosterone by these cell preparations offering further indication of biological activity (Fig. 3).

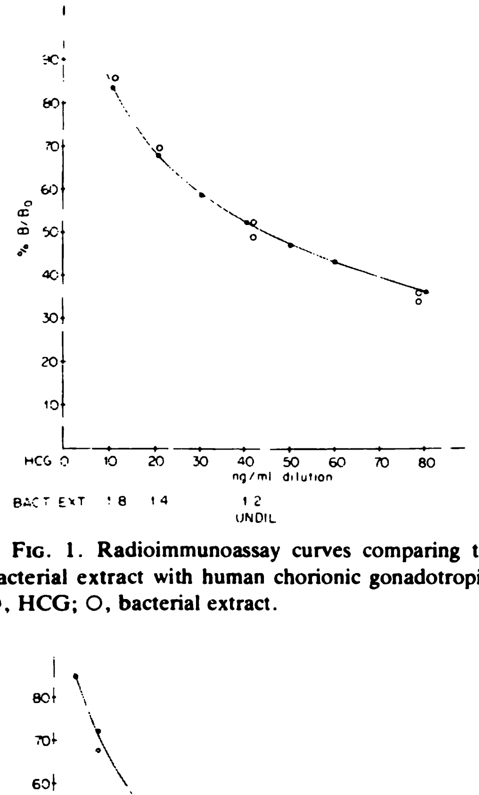

FIG. 1. Radioimmunoassay curves comparing the bacterial extract with human chorionic gonadotropin. ●, HCG; O, bacterial extract.

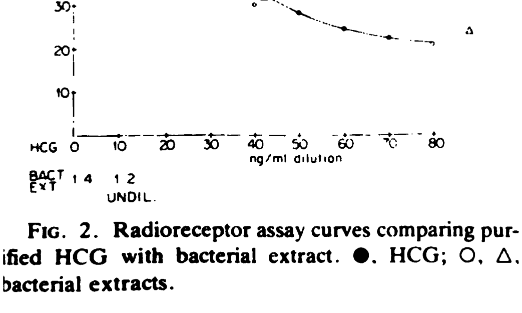

FIG. 2. Radioreceptor assay curves comparing purified HCG with bacterial extract. ●, HCG; O, △, bacterial extracts.

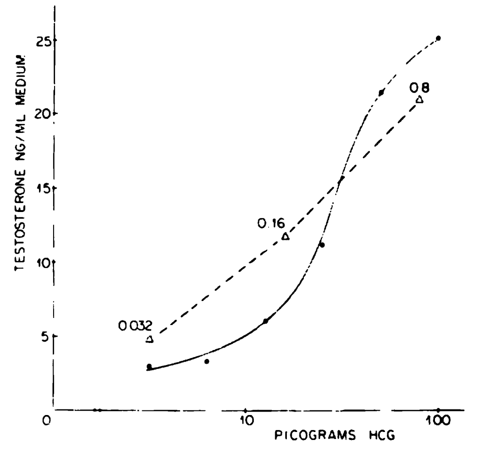

FIG. 3. Effect of purified HCG and bacterial extract on the production of testosterone by isogated mouse Leydig cell preparations. - - -△- - -△, Bacterial extracts (milliliter of extract); —●——●, purified HCG (picograms).

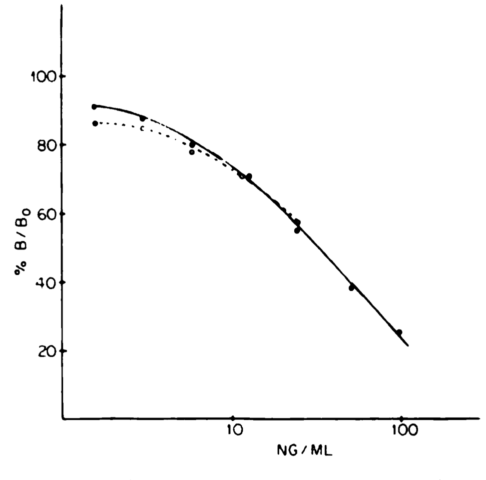

FIG. 4. Radioreceptor assay comparison of purified HCG with the 0.2 *M* methyl-alpha-D-mannopyranoside eluate, of the bacterial extract chromatographed on concanavalin A-Sepharose column. ——, HCG; -----, bacterial extract.

These parameters (i.e., immunological and biological) characteristic of different conformational areas of the protein molecule present strongly convincing evidence for the similarity between the bacterial material and HCG.

Treatment of bacterial HCG with trypsin destroyed 38% of the activity as measured

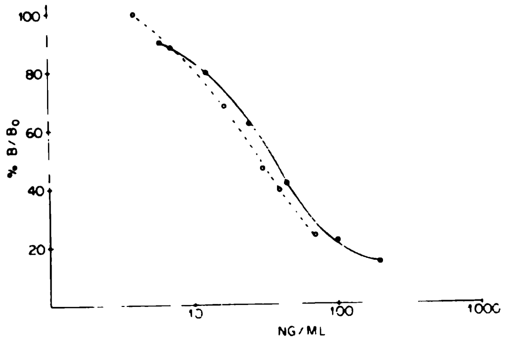

FIG. 5. Radioimmunoassay comparison of purified HCG with the 0.2 *M* methyl-alpha-D-mannopyranoside eluate, of the bacterial extract chromatographed on concanavalin A-Sephadex column. -----, bacterial extract; ——, HCG.

by RIA, a phenomenon also noted with purified HCG, and is indicative of the protein nature of the material. The probability that this substance is a glycoprotein is manifest in its adsorption onto Concanavalin A-Sepharose columns (Figs. 4, 5) from which 75% of the adsorbed bacterial extract could be eluted only with 0.2 *M* methyl alpha-D-mannopyranoside.

It should be pointed out that although the organism isolated from urine of patient A. K. elaborated HCG-like material when cultured, we were unable to demonstrate HCG in this urine by either RRA or RIA. It is also important to note that the amount of "HCG-like material" synthesized in culture is quite small and unpredictable, even when the same organism is employed. However, development of optimum culture conditions may remedy these difficulties and permit further work toward isolation, purification, and characterization of this substance.

We believe that the Livingston findings (1) are the first indication of the possible synthesis of a mammalian protein hormone by a microbial organism and imply either exchange of genetic material or the presence in microrganisms of genetic material capable of mediating such synthesis under particular conditions. However, until enough material is isolated and purified to enable work on structural and chemical properties, conclusions of strict identity should be withheld.

Summary. Our data tend to confirm the observations of Livingston *et al.* (1) that a microorganism(s) isolated from the urine of cancer patients is capable of producing a human chorionic gonadotrophinlike substance. Chromatographic properties as well as immunologic and receptor assay techniques offer strong evidence for this similarity.

We are indebted to Dr. K. Sundaram for his examination of the extracts for *in vitro* gonadotrophic activity, and to Drs. M. Goore and N. Starkovsky for their aid in performing the radioimmunoassay and radioreceptor assay. Also, to Dr. Livingston for supplying us with cultures of the organism as well as samples of her isolates.

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GS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 411-414 (1976)

Vitamin A Prevention of Triamcinolone Acetonide Effects on Granuloma Growth: Lack of Effect on Prolyl Hydroxylase[1] (39408)

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: continued extensive use of potent osteroids necessitates an understand- the mechanism of action of this class ıgs. Clinically, some of the side effects are the result of altered collagen me- sm are: ecchymoses, atrophy, telan- sia, and stria. While there have been investigations concerning clinical and emical parameters of glucocorticoid ›y, the mechanism(s) of these phe- na to date remain unclear. Ragan *et al.* :monstrated a decrease in granuloma- issue in ear wounds of rabbits adminis- cortisone acetate. Other investigators demonstrated antianabolic effects of :orticoids on collagen metabolism (2-

lich and Hunt (14) reported that vi- A overcame the inhibitory effects of ›ne on tensile strength in the early of healing. These workers also re- l that the reduction of inflammatory ıfiltration, fibroplasia, capillary bud- and deposition of collagen fibers fol- ; glucocorticoid administration is re- l by concurrent vitamin A therapy These studies suggested that vitamin ninistration reversed the antianabolic ; of glucocorticoids on collagen syn-

ninistration of glucocorticoids to rats ; in a decrease of prolyl hydroxylase in .l tissues and in sponge-induced granu- ›us tissue (16-18). The present studies were undertaken to determine if concurrent vitamin A treatment would reverse the effect of glucocorticoid on prolyl hydroxylase activity in granulomatous tissue.

Materials and methods. 4-[^{3}H]Proline (15 Ci/mmole) and 2,3-[^{3}H]proline (30 Ci/mmole) were obtained from New England Nuclear. Polyvinyl sponges (3.8 × 1.5 cm) were purchased from Scientific Products. Triamcinolone acetonide (T.A.) and vitamin A were obtained from Squibb & Co. and USV Pharmaceuticals, respectively. Eagle's minimal essential medium with Hanks' balanced salt solution (pH 7.4) 2 × glucose and 2 × $NaHCO_3$ and penicillin-streptomycin solution were obtained from Grand Island Biologicals.

Four-week-old male Sprague-Dawley rats (100 g) were anesthetized with diethyl ether. All operations were carried out using sterile technique. A sterilized sponge was implanted in the dorsothoracic region using a trocar which was inserted into an incision 2 cm above the tail. The trocar was removed and the wound was closed with stainless steel autoclips. Immediately after sponge implantation, intraperitoneal administration of drugs was initiated. Animals received daily injections for the next 3 days. Six to eight rats per group were treated with either 0.2 ml of 0.9% (w/v) saline, T.A. (20 mg/kg), vitamin A (150,000 I.U./kg) or T.A. plus vitamin A. The control group was injected with the vehicle. The animals were killed on Day 4, and the tissue-covered sponges were dissected free from the surrounding tissues. At autopsy, there were no visible signs of infection of granulomatous tissue. The granulomatous tissue was stripped away from the sponge, weighed, frozen in liquid nitrogen, pulverized, and a 10% (w/v) tissue homogenate in 0.25 *M*

ıs investigation was supported by United Public Service Grants No. AM05586 and No. 16 from the National Institute of Arthritis, Me- ı, and Digestive Diseases.

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sucrose was prepared using the polytron ST system. The granuloma represents only the tissue surrounding the sponge. The 16,000 *g* supernatant was prepared and used to measure enzyme activity by a modification (18) of the method of Hutton *et al.* (19). An aliquot of the original homogenate was hydrolyzed in 6 *N* HCl and assayed for hydroxyproline content by the method of Stegeman and Stalder (20). Total protein was determined by the method of Lowry *et al.* (21). Deoxyribonucleic acid content was quantified by the method of Burton (22). Additional aliquots were extracted with chloroform-methanol for lipid content (23).

The rate of proteinaceous hydroxyproline formation in granulomatous tissue was determined by a modification of an *in vitro* tissue mince system previously described (24). The granulomatous tissue was rinsed twice in minimum essential medium (Eagle, F-12) containing 10,000 units of penicillin and 10,000 μg of streptomycin. Samples of granulomatous tissue from the animals were incubated in 10 ml of minimal essential medium which was made 0.28 m*M* with sodium ascorbate. 2,3-[^{3}H]Proline (500 μCi) were added and incubated under air at 37° for 60 min. After incubation each mixture was separated from the media by filtering through two layers of cheesecloth. The tissue was rinsed twice with 25 ml of 10% (w/v) trichloroacetic acid (TCA) at 0–3° and homogenized in 10% (w/v) TCA with a Polytron-ST homogenizer for 45 sec. The sample was centrifuged at 10,000 *g* for 15 min. The tissue pellet was resuspended and washed three times with 5% TCA. Ten milliliters of 6 *N* HCl was added to the tissue pellet and the sample was hydrolyzed for 18 hr in an autoclave. Hydroxyproline was isolated by Dowex cationic-exchange chromatography as described previously (18). The formation of proteinaceous hydroxyproline was linear for at least 90 min.

Results. A dose of 150,000 I.U./kg of vitamin A was required to prevent the weight loss of granuloma tissue in animals treated with 20 mg/kg T.A. (Table I). The amounts of DNA, protein, and proteinaceous hydroxyproline per granuloma were reduced after T.A. treatment when expressed per total granuloma (Table II). Since these differences were not observed when the data were expressed either on a DNA or protein basis, the decreases in DNA, protein, and hydroxyproline result from the general antianabolic effect of glucocorticoid on granuloma growth. Vitamin A treatment prevented the reduction of DNA, protein, and hydroxyproline contents. The amount of lipid per granuloma in both T.A. and T.A. + vitamin A groups was twice as much as either saline control or vitamin A alone. Concurrent vitamin A

TABLE I. THE DOSE OF VITAMIN A REQUIRED TO REVERSE THE TRIAMCINOLONE-MEDIATED DECREASE OF GRANULOMA WEIGHT.[a]

Treatment	Granuloma weight (mg)
Saline	707 ± 66
Vitamin A (50,000 I.U./kg)	746 ± 14
Vitamin A (150,000 I.U./kg)	788 ± 88
T.A.	411 ± 40*
T.A. + vitamin A (50,000 I.U./kg)	530 ± 55*
T.A. + vitamin A (150,000 I.U./kg)	887 ± 143

[a] The values represent the mean ± SE of four to eight samples. Animals were either treated with saline, vitamin A at the doses indicated, triamcinolone (T.A. 20 mg/kg), or vitamin A plus T.A. for four daily injections. The granulomatous tissue was dissected from the animal and weighed as described in the text.

* Significantly different from control at $P \leq 0.05$.

TABLE II. VITAMIN A REVERSAL OF GRANULOMA WEIGHT, HYDROXYPROLINE, DNA, AND PROTEIN CONTENT IN TRIAMCINOLONE-TREATED RATS.[a]

Treatment	Δ Body weight (g)	Granuloma weight (g)	Hydroxyproline (μg)	DNA (μg)	Protein (mg)	Lipid (mg)
Saline	+ 35 ± 1.9	0.85 ± 0.6	1.79 ± 0.26	1.18 ± 0.17	60.0 ± 5.8	18.9 ± 3.7
Vitamin A	+ 27 ± 4.2	0.84 ± 0.10	1.92 ± 0.27	1.08 ± 0.14	64.3 ± 9.2	19.9 ± 3.4
T.A.	− 17 ± 1.6*	0.29 ± 0.03*	0.67 ± 0.12*	0.51 ± 0.10*	22.0 ± 1.16*	31.2 ± 3.8**
Vitamin A plus T.A.	− 20 ± 1.5*	0.69 ± 0.07	1.52 ± 0.18	1.51 ± 0.04**	56.5 ± 3.78	32.2 ± 2.1**

[a] Values represent the mean ± SE of four to eight samples. Triamcinolone acetonide (T.A. 20 mg/kg) and vitamin A (150,000 I.U./kg) were injected ip for 4 consecutive days from the day of sponge implantation. The granuloma tissues were prepared and assayed for protein, DNA, and hydroxyproline content as described in the text. Hydroxyproline, DNA, and protein values represent the amounts per granuloma.

* Significantly lower than control at $P \leq 0.05$.

** Significantly greater than control at $P \leq 0.05$.

nent did not prevent the reduction of weight by T.A.

e effects of vitamin A treatment on ȝen synthesis of granuloma tissue fol- ıg T.A. treatment was quantified by uring both prolyl hydroxylase activity proteinaceous hydroxyproline forma- The prolyl hydroxylase activity was ficantly decreased from the control in both T.A. and T.A. + vitamin A ɔs (Table III). Concurrent vitamin A nent did not alter the effect of T.A. on l hydroxylase activity. The T.A. group ıot significantly different from the vi- ı A plus T.A. group. Proteinaceous ıxyproline formation was decreased in T.A. and T.A. plus vitamin A groups e III). The steroid-induced decrease of prolyl hydroxylase activity and the rate ɔteinaceous hydroxyproline formation ınulomatous tissue was not altered by ırrent vitamin A treatment.

cussion. The treatment of either ds or sponge-induced granuloma with osteroids affects several parameters of eration and repair including reduction ammatory cell infiltration, fibroplasia, ollagen synthesis (2–13). In the pres- ıdy a reduction in weight and vascular- the granuloma tissue was noted in the d-treated animals, as previously re- l (3–5, 7, 8). Concurrent vitamin A prevented the decreases of several parameters by T.A. Although granuloma weight and the amounts of DNA, protein, and proteinaceous hydroxyproline were all significantly lower than control values in the T.A. group, all of these parameters returned to control levels when vitamin A was given concurrently.

.E III. PROLYL HYDROXYLASE ACTIVITY AND OXYPROLINE FORMATION IN GRANULOMATOUS UE OF RATS TREATED WITH TRIAMCINOLONE AND/OR VITAMIN A.[a]

nent	Prolyl hydroxylase activity (dpm × 10^{-3}/mg protein)	[^{3}H]Hydroxyproline formed (dpm × 10^{-4}/mg protein)
	6.3 ± 1.3	18.8
ı A	5.3 ± 1.5	26.0
	1.9 ± 0.7[*]	3.4
ı A .A.	0.6 ± 0.3[*]	5.2

ues for prolyl hydroxylase activity represent n ± SE of six samples. Triamcinolone (T.A. 20 ınd vitamin A (150,000 I.U./kg) were injected consecutive days. The granulomatous tissue sed and prepared for assay of prolyl hydroxyl- ity as described in the text. Samples of granu- s tissue from the rats of each group were ınd incubated *in vitro* with [^{3}H]proline as de- n the text.

ıificantly different at $P \leq 0.05$.

Glucocorticoid administration to rats results in a decreased prolyl hydroxylase in both normal and granulomatous tissues (16–18). The dose of T.A. used significantly decreased prolyl hydroxylase activity in granulomatous tissue. Although Stein and Keiser (25) demonstrated that vitamin A is able to prevent the glucocorticoid-mediated decrease of this enzyme in wounded skin, we did not observe this in sponge-induced granulomatous tissue. Both prolyl hydroxylase activity and the rate of proteinaceous hydroxyproline formation were decreased in animals treated with glucocorticoid plus vitamin A. The dose of vitamin A was the lowest dose which prevented the granulomatous weight reduction by T.A.

Our data indicate that concurrent vitamin A treatment prevented the effect of this steroid on granuloma vascularity and weight but did not block the specific effects of T.A. on prolyl hydroxylase activity and hydroxyproline formation. The degree of vascularity was visually estimated at autopsy. The decrease in proteinaceous hydroxyproline formation in T.A. plus vitamin A groups may represent either a decrease in collagen polypeptide synthesis and/or prolyl hydroxylation step of collagen biosynthesis. Although hydroxyproline formation was decreased in the vitamin A plus T.A. group, total granulomatous proteinaceous hydroxyproline was the same as control. This is quite possible since the decrease in prolyl hydroxylase in tissues occurs after several daily steroid injections (17), whereas the decrease in protein synthesis is evident within hours after initiation of glucocorticoid treatment (7, 8, 10–12, 18, 26). A longer treatment period may be required to observe decreases in total proteinaceous hydroxyproline.

The data indicate that although vitamin A is able to prevent the antiinflammatory activity of glucocorticoids, this vitamin does not block the specific effect of glucocorticoids. The data suggest that the antiinflammatory activity of this class of drugs is not mediated through a selective inhibition of collagen synthesis.

Summary. Vitamin A administered concurrently with triamcinolone acetonide prevented the effects of steroid on granuloma wet weight, vascularity, hydroxyproline, protein, and DNA. Although vitamin A blocked the general inhibitory effects of glucocorticoid treatment on granuloma growth, decreases of both prolyl hydroxylase activity and proteinaceous hydroxyproline formation were observed. These results indicate that vitamin A did not prevent the specific effects of glucocorticoids on prolyl hydroxylase activity and hydroxyproline formation.

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DINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 415–418 (1976)

ect of Ca^{2+} Binding by EGTA on Renin Release in the Isolated Perfused Rabbit Kidney[1] (39409)

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/hether or not ionized calcium (Ca^{2+}) is ictor in renin release is still unclear. helakis (1) showed that renin release n kidney slices was depressed when cal- n was omitted from the incubation fluid, was maximal when the concentration of ium in incubation media was increased .5 m*M*/liter. Kotchen *et al.* (2), how- , found that the acute injection of cal- n into the renal artery of a dog sup- sed renal venous PRA, and that PRA renal renin content were decreased in during chronic administration of cal- n. Previous studies from this laboratory d to find any change in PRA during e EDTA-induced hypocalcemia in nor- subjects or subjects with mild renal fail- (3), or during calcium infusions in sub- s with varying degrees of renal failure

i studies in the intact animal or human), where simultaneous changes in ex- nal and renal factors modulating renin ase cannot be prevented, calcium could retically affect renin release by one or a bination of several mechanisms: (a) it it affect circulating catecholamines (5) h in turn could alter renin secretion (6); it might have a direct action on intra- l beta-adrenergic renin receptors via its wn action on cyclic AMP (7); (c) it it affect urinary sodium excretion (8), thus changing sodium delivery to the macula densa as proposed by Kotchen *et al.* (2); and (d) calcium-induced changes in systemic and renal hemodynamics (9–10) might alter renin release.

The use of an isolated perfused kidney eliminates the possibility of changes in circulating catecholamines and enables maintenance of a constant perfusion pressure, permitting analysis of the intrarenal factors of renin release. Therefore, this model was utilized to study the effects of reduction of Ca^{2+} during stimulation with isoproterenol and theophylline.

Methods. Details of the isolated, perfused rabbit kidney model used in our laboratory has been described previously (11, 12). Each experiment consisted of two consecutive periods of 10 min each, a control period followed by an infusion period of known stimuli of renin release, isoproterenol (13, 14), or theophylline [theophylline ethylene diamine (1-3 dimethylxanthine)] (15). Both agents were administered into the renal artery during the first 8 min of the infusion period. The infusion rate of isoproterenol was 0.01 μg/kg/min, and that of theophylline was 7 mg/kg/8 min.

Two sets of experiments were performed. In the control group (Group A) the kidney was perfused with ,fresh rabbit blood; in Group B, EGTA [ethylene glycol bis(beta-aminoethyl ether) *N*,*N*,tetraacetic acid] was added to the blood perfusate in a concentration of 1 m*M*, calculated to bind essentially all of the circulating Ca^{2+} (7). The addition of EGTA to fresh rabbit blood *in vitro* in equimolar concentration to that of the experiments did not alter the velocity reaction of the hydrolyses of hog renin substrate by rabbit renin (Lineweaver–Burk

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plot). In six duplicate studies of unaltered rabbit serum compared to rabbit serum with EGTA added prior to incubation, radioimmunoassay of Angiotensin I yielded similar results.

In all experiments perfusion pressure was kept constant at 80 ± 3 mm Hg. Renal venous blood specimens were collected at the end of each 10-min period for measurement of serum renin concentration (SRC), and samples were drawn from the renal artery at each midperiod for determination of plasma creatinine and serum electrolytes. Urine collections were made during the theophylline experiments. Methods for determination of SRC, electrolytes, creatinine, glomerular filtration rate, and renal blood flow have been described previously (11, 12). Student's *t* test for paired data was used for statistical analysis.

Results. Effect of EGTA on isoproterenol-stimulated renin secretion (Fig. 1). In the control experiments, Group A, isoproterenol caused a 17% increase of renal vein serum renin concentration ($P < 0.001$). A similar increase was observed during perfusion with EGTA-containing blood, Group B. Renal vascular resistance and serum potassium did not change significantly during the control period, during isoproterenol administration, or following the addition of EGTA.

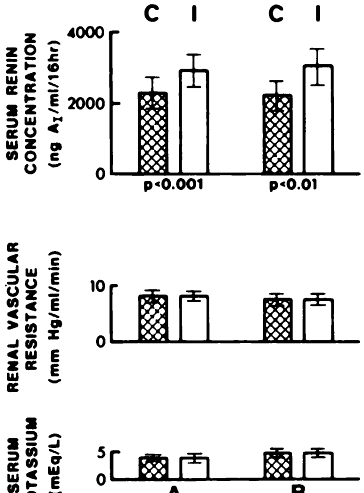

FIG. 1. The influence of isoproterenol (I) on renal vein serum renin concentration, renal vascular resistance, and serum potassium, as compared to a preceding 10-min control period (C). Perfusate in A is rabbit blood ($n = 15$); and in B is rabbit blood with 1 m*M* EGTA ($n = 6$).

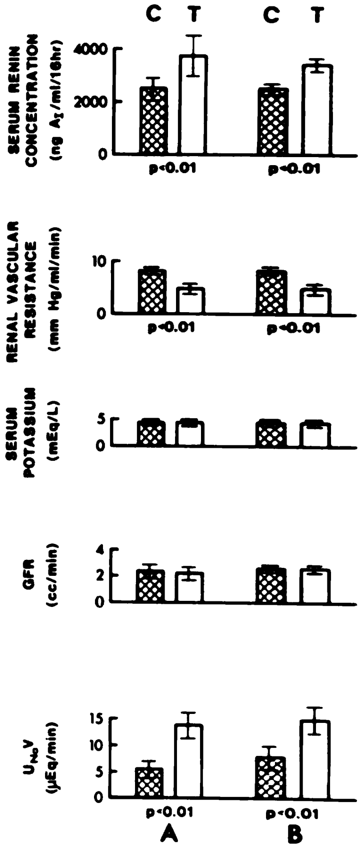

FIG. 2. The influence of theophylline (T) on renal vein serum renin concentration, renal vascular resistance, serum potassium, GFR, and $U_{Na}V$ as compared to a preceding 10-min control period. Perfusate in A is rabbit blood ($n = 6$); and in B is rabbit blood with 1 m*M* EGTA ($n = 6$).

Effect of EGTA on theophylline-stimulated renin secretion (Fig. 2). Renal vein serum renin concentration increased significantly ($P < 0.01$) during theophylline infusion in group A. The addition of EGTA (Group B) did not change the theophylline-induced renin release (N.S.). During infusion of theophylline, renal vascular resistance decreased from 8.1 to 5.0 mm Hg/ml/min ($P < 0.01$) without EGTA, and a similar decrease was observed when EGTA was added. GFR and serum potassium did not change during theophylline infusion or with the addition of EGTA. Urinary sodium excretion increased from 5.5 ± 1.5 during the control period to 14 ± 2.5 μequiv/min during theophylline infusion ($P < 0.01$) without EGTA. A similar increase was observed when EGTA was added.

Discussion. Renin secretion by the kidney is regulated by an interrelated complex system, mediated by changes in renal perfusion pressure (16), renal resistance (17), urinary sodium excretion (16–19), serum potassium (20), sympathetic nerve stimulation (5, 21), and catecholamines (5, 6). The presence of abundant adrenergic nerve fibers in close proximity to the juxtaglomerular cells (22) and the fact that both catecholamines and cyclic AMP cause renin release when added to the kidney *in vitro* (16) has led to the assumption that catecholamines increase renin secretion by direct action on β-adrenergic receptors in the renin-secreting cells, mediated by cyclic AMP.

Cyclic AMP content is generally controlled by adenylcyclase, which increases cyclic AMP production, and phosphodiesterase activity, which decreases cyclic AMP content. In smooth muscle, reduction of external free Ca^{2+} increases phosphodiesterase activity with a resultant decreased cyclic AMP content (7). This might explain the decreased renin release by kidney slices *in vitro*, when calcium was omitted from the incubation fluid reported by Michelakis *et al.* (1). The influence of binding the free Ca^{2+} by EGTA was therefore examined when the perfused rabbit kidney was stimulated by isoproterenol, which activates adenyl cyclase, or by theophylline, which inhibits phosphodiesterase activity. Both of these stimuli should theoretically increase cyclic AMP.

The observations in the present study show that in the isolated kidney the increase of renal vein SRC during stimulation with isoproterenol and theophylline was unaffected by binding of free Ca^{2+} with EGTA. This does not exclude the possibility that in different circumstances changes in sodium excretion secondary to changes in filtered calcium may alter renin release (8). In the present experiments control $U_{Na}V$ was uneffected by the addition of EGTA, and the marked naturesis secondary to theophylline administration in both groups A and B could have masked any decrease in urinary sodium secondary to calcium-binding by EGTA.

Vandongen and Peart (23) studied renin secretion in the isolated, perfused rat kidney and observed that elimination of calcium from and the addition of EDTA to the perfusion fluid increased noradrenaline-induced renin release, as did the alpha-adrenergic blocking agent, phenoxybenzamine. They postulated that intrarenal vascular contractility and inhibition of renin secretion after alpha-adrenergic receptor activity may be related and may be calcium-dependent. In the present experiments, binding of the perfusate Ca^{2+} did not affect renin release secondary to direct beta-adrenergic stimulation (isoproterenol) or to changes in vascular reactivity (theophylline), two major mechanisms of renin release.

Summary. In the isolated, perfused rabbit kidney, binding of ionized calcium by EGTA did not affect the increased renin release secondary to two well-known stimuli, theophylline and isoproterenol. These results indicate that Ca^{2+} is not a major factor in the intrarenal regulation of renin secretion.

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Effects of Relaxin on Early Pregnancy in Rats (39410)

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polypeptide hormone, relaxin, pre- he reproductive tracts for parturition :ral mammalian species. In rats, re- ; secreted by the ovaries during the ek of a 22-day pregnancy (1), and its bvious effect is to soften and dilate rine cervix (2). When rats are ovari- zed on Day 15 of gestation, preg- nay be maintained by daily injections esterone and estradiol. However, the fails to soften, placental detachment y, and parturition is impaired unless is administered together with the hormones (2).

ice, in addition to a gradual softening cervix, a long interpubic ligament luring the last week of a 20–21-day n period and the uterus becomes responsive to oxytocin (3). These s correlate with increased blood lev- relaxin (1). As in rats, there is a of parturition in ovariectomized nt mice unless relaxin therapy is insti- 2).

effects of relaxin on early pregnancy ot been documented. It seemed possi- at such early administration might bout a very premature "parturition." present study we have injected re- nto rats in the peri-implantation pe- n addition, we have investigated the of relaxin on the response of the nt rat to subabortifacient doses of glandin $F_{2\alpha}$ ($PGF_{2\alpha}$), another sub- involved in the initiation of parturi- -7).

rials and methods. Animals. Charles CD or Marland Breeding Farms MFS male rats (both Sprague-Dawley-de- were used. Pregnancies were timed suppliers (midnight ± 6 hr) and the s were delivered to our laboratory on or 2 of pregnancy. Day 1 is the g on which spermatozoa are found in inal smear.

none and drug treatments. Swine ovarian relaxin preparations (8) ranging in potency from 1000 to 3000 units (9) per mg were suspended in a 5% beeswax in peanut oil vehicle and injected sc at the doses indicated in the text and tables. Groups of control rats were injected sc with equivalent milligram doses of bovine serum albumin (BSA) or a nonrelaxin polypeptide fraction prepared from swine ovaries in the beeswax-oil suspension. Progesterone was injected sc in sesame oil. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was injected sc in 0.1 *M* phosphate buffer (pH 7.4). Dexamethasone and indomethacin were administered by gavage as aqueous suspensions.

Experimental. Relaxin or control preparations were administered sc as single doses on Days 3, 4, or 5 of pregnancy or as multiple doses on Days 3–5 or 4 and 5 in rats. Progesterone was injected sc on Days 3–6 or 2–9 of pregnancy in rats. Dexamethasone was administered orally on Days 4–6. Indomethacin was given by gavage days 3–6. $PGF_{2\alpha}$ was injected sc as a single dose on Day 5 of pregnancy alone or in combination with relaxin. In all experiments, rats were killed on Day 10 and living fetuses, resorptions, and total implantation sites were counted. In one experiment blood was drawn for progesterone determination on Day 6, 24 hr after a single sc injection of relaxin. The rats were then killed on Day 9 and the uterine contents observed as above.

Progesterone radioimmunoassay. Serum progesterone levels were determined by the method of Abraham *et al.* (10) as modified in our laboratory (11).

Results. Injection of 0.5 (but not 0.1) mg of relaxin sc daily on Days 3–5 of pregnancy induced resorption of a large number of fetuses (Table I). This effect was not duplicated by 0.5-mg daily doses of bovine serum albumin (BSA) or a nonrelaxin polypeptide fraction of sow ovaries obtained as a by-product during the extraction of relaxin (Table I).

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served.

Single doses of 1 mg of relaxin administered on Day 4 or 5, but *not* on Day 3, likewise terminated pregnancy in rats (Table II). Progesterone, but not indomethacin on Days 3–5, prevented termination of pregnancy by relaxin on Day 5 (Table II). Similarly, resorption of litters induced by 0.5-mg doses of relaxin on Days 4 and 5 was prevented by daily injections of progesterone from the second to the ninth day of pregnancy (Table II). Injections of 10 or 30 μg of dexamethasone on Days 4, 5, and 6 partially or completely reversed the adverse effects of relaxin on litter survival (Table III).

Smaller daily doses of relaxin (0.1 mg) did not induce resorptions (Table IV). A single injection of 1 mg of $PGF_{2\alpha}$ on Day 5 of pregnancy likewise failed to affect the course of pregnancy (Table IV). However, when 1 mg of $PGF_{2\alpha}$ was injected on Day 5 following 0.1-mg doses of relaxin on Days 3, 4, and 5, nearly complete resorption of litters was observed (Table IV).

Serum progesterone levels were found to be similar to those of controls 24 hr after single injections of 3 mg of partially purified or 1 mg of highly purified relaxin (Table V).

Discussion. Two or three injections of relatively large doses of purified porcine relaxin (0.5–1.0 mg) around the expected time of implantation terminated pregnancy in rats. However, this effect appeared to be specific for the relaxin hormone per se as it was not duplicated by injections of nonrelaxin polypeptides obtained from pregnant sow ovaries or injection of a foreign protein such as BSA. Single injections of 1 mg of relaxin on Day 4 or 5, but not on Day 3, likewise terminated pregnancy. In no case did relaxin interfere with implantation. The uterine contents were protected against the effects of relaxin by injections of progesterone or dexamethasone. The latter steroid

TABLE I. EFFECTS OF RELAXIN ON EARLY PREGNANCY IN RATS.

Treatment[a] (Days 3–5)	No. rats	Av no. living fetuses[d]	Av no. resorptions[d]	Av no. implants[d]
Control	6	12.3 ± 1.1	0.4 ± 0.2	13.5 ± 1.2
Relaxin[b] 0.5 mg	5	2.6 ± 2.6[c]	8.0 ± 2.8[c]	10.6 ± 2.1
Relaxin[b] 0.1 mg	5	11.2 ± 0.8	0	11.2 ± 0.8
Control ovarian extract 0.5 mg	6	13.2 ± 0.8	0	13.2 ± 0.8
BSA 0.5 mg	6	10.0 ± 3.0	0	10.0 ± 3.0

[a] In 0.2 ml of 5% beeswax in peanut oil.
[b] 21 AAE relaxin, 3000 units/mg.
[c] $P < 0.02$ versus control.
[d] Determined at autopsy on Day 10 of pregnancy.

TABLE II. EFFECT OF SINGLE DOSES OF RELAXIN ON EARLY PREGNANCY IN RATS AND THE ANTIABORTIFACIENT ACTION OF PROGESTERONE.

Treatment[a]	Injection on Day:	No. rats	Av no. living fetuses	Av no. resorptions[d]	Av no. implants[d]
Control	–	7	10.6 ± 2.7	0	10.6 ± 2.7
Relaxin[b] 1 mg sc	3	6	10.8 ± 1.4	1.8 ± 0.8	12.6 ± 0.7
Relaxin 1 mg sc	4	7	1.8 ± 1.5[c]	8.8 ± 2.7[c]	10.7 ± 2.2
Relaxin 1 mg sc	5	6	0.5 ± 0.5[c]	8.3 ± 2.9[c]	8.8 ± 2.9
Relaxin 1 mg sc + Progesterone 10 mg sc	5 3–6	4	12.5 ± 0.6	0	12.5 ± 0.6
Relaxin 1 mg sc + Indomethacin 0.5 mg po	5 3–6	4	1.5 ± 1.5	10.5 ± 3.4	12.0 ± 3.2
Relaxin 0.5 mg sc	4, 5	6	1.7 ± 1.7[c]	7.2 ± 2.8[c]	8.8 ± 2.5
Relaxin 0.5 mg sc + Progesterone 5 mg sc	4, 5 2–9	6	10.1 ± 1.4	1.6 ± 0.8	11.8 ± 0.8

[a] In 0.2 ml of 5% beeswax in peanut oil.
[b] Relaxin preparation 21 AAE (3000 U/mg).
[c] Significantly different from control, $P < 0.01$.
[d] Determined at autopsy on Day 10 of pregnancy.

TABLE III. REVERSAL OF RELAXIN-INDUCED RESORPTIONS BY DEXAMETHASONE IN RATS.

Treatment	No. rats	Living fetuses[c]	Resorptions[c]	Total implants[c]
Control	10	12.4 ± 0.5	0.1 ± 0.1	12.5 ± 0.5
Relaxin[a] 0.5 mg sc Days 4 and 5	10	1.6 ± 1.0[b]	11.0 ± 1.1[b]	12.6 ± 0.6
Relaxin[a] 0.5 mg sc Days 4 and 5 + Dexamethasone 10 μg po Days 4-6	5	6.8 ± 3.0	3.0 ± 1.5	9.5 ± 2.2
Relaxin[a] 0.5 mg sc Days 4 and 5 + Dexamethasone 30 μg po Days 4-6	5	11.2 ± 0.7	0.2 ± 0.2	11.4 ± 0.4
Dexamethasone 10 μg po Days 4-6	5	12.6 ± 1.2	0	12.6 ± 1.2
Dexamethasone 30 μg sc Days 4-6	5	13.2 ± 0.4	0	13.2 ± 0.4

[a] Relaxin preparation No. *15 AAE* (1000 units/mg) in 0.2 ml of 5% beeswax in peanut oil.
[b] Significantly different from control, $P < 0.01$.
[c] Determined at autopsy on Day 10 of pregnancy.

TABLE IV. EFFECTS OF RELAXIN AND $PGF_{2\alpha}$ ON PREGNANCY IN RATS.

Treatment	Days	No. rats	Fetuses	Resorptions[c]	Total implants[c]
Control (BSA 0.5 mg sc)	3-5	5	12.0 ± 2.8	0	12.0 ± 2.8
Relaxin[a] 0.1 mg sc	3-5	5	11.2 ± 0.8	0	11.2 ± 0.8
$PGF_{2\alpha}$ 1 mg sc	5	5	13.6 ± 1.2	0.8 ± 0.4	14.5 ± 1.3
Relaxin 0.1 mg sc + $PGF_{2\alpha}$ 1 mg sc	3-5 5	5	1.2 ± 1.2[b]	8.4 ± 2.5[b]	9.6 ± 2.6

[a] Relaxin preparation No. *21 AAE* (3000 units/mg) in 0.2 ml of 5% beeswax in peanut oil.
[b] Significantly different from control, $P < 0.01$.
[c] Determined at autopsy on Day 10 of pregnancy.

TABLE V. SERUM PROGESTERONE LEVELS 24 HR AFTER RELAXIN INJECTION IN EARLY PREGNANT RATS.

Treatment[a]	Daily dose (mg)	Days of injection	No. rats	Av no. living fetuses[b]	Av no. resorptions[b]	Serum progesterone (ng/ml)[c]
None	–	–	4	13.5	0	66.7 ± 2.6
Relaxin (15 AAE)	3.0	4, 5	3	0	8.6	75.7 ± 12.8
Relaxin (21 AAE)	1.0	5	5	5.5	7.8	68.5 ± 10.3

[a] Injected in 0.2 ml of 5% beeswax in peanut oil.
[b] Observed on Day 9 of pregnancy.
[c] Blood drawn on Day 6 of pregnancy.

has no progestational activity but corticosteroids may induce secretion of prolactin (12, 13). Prolactin, in turn, would increase progesterone synthesis by the ovaries. It is also possible that dexamethasone, by virtue of its antiinflammatory activity, may somehow directly antagonize the effects of relaxin. (Relaxin is known to induce inflammatorylike changes in permeability and composition of the uterus; 14). However, a nonsteroidal antiinflammatory agent, indomethacin did not prevent the abortifacient effects of relaxin.

Relaxin and $PGF_{2\alpha}$ in doses which separately had no adverse effects on pregnancy, induced resorption of litters when given in combination. We had considered the possibility that relaxin may exert its action by inducing prostaglandin synthesis. However, the failure of a prostaglandin synthetase inhibitor, indomethacin to prevent the abortifacient action of relaxin makes this appear unlikely. It does seem possible that relaxin and PGs may act synergistically at term to bring about parturition.

The mechanism(s) whereby large doses of relaxin terminate pregnancy is not known. The hormone did not appear to alter serum progesterone levels and yet exogenous progesterone antagonized the abortifacient action of relaxin. This finding suggests that the effect of relaxin may be exerted on the uterus inasmuch as this organ is the major site of action of progesterone in maintaining pregnancy. Relaxin may inhibit decidualization (15). Relaxin also is known to affect the composition (14), structural framework (16, 17), and motility (e.g., 17) of the uterus. Relaxin may be able to reverse the progesterone "block" to the propagation of coordinated uterine contractile activity.

Summary. Administration of relaxin in the peri-implantation period terminated pregnancy in rats. The effect was blocked by progesterone and dexamethasone but not by indomethacin. When relaxin and $PGF_{2\alpha}$ were given concomitantly in doses which singly did not interfere with pregnancy, resorption of fetuses was observed. It is suggested that relaxin may antagonize the progesterone "block" to coordinated uterine contractions.

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EDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 423–427 (1976)

rsistence of the Renal Response to Atrial Tamponade after Cardiac Denervation[1] (39411)

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revious studies in our laboratory demon- ted that decreases in renal sodium excre- i and urine flow are elicited when atrial ısmural pressure is reduced in the dog by infusion of isotonic saline into a specially structed pericardial pouch which en- es the atria only (1, 2). This procedure ial tamponade) produced no significant nges in left atrial pressure or aortic pres- (2). Consequently, we suggested that changes in salt and water excretion are ited reflexly by the changes in atrial ısmural pressure (1, 2) although other sibilities were considered (1).

ı later experiments we found that central ous pressure consistently is increased by roximately 2–3 mm Hg during atrial ponade (3), thus indicating that this pro- ure does cause distinct, though subtle, nges in systemic hemodynamics. The nges in renal excretion that occur during ıl tamponade therefore conceivably ld be initiated either by changes in atrial ptor activity or by extra-atrial changes stemic hemodynamics. In an attempt to erentiate between these two possibili- , we performed a series of experiments gned to determine the effect of atrial ponade on renal function after known rent pathways from the heart had been rrupted. The interruption of cardiac af- nt pathways would be expected to abol- or attenuate the renal response if it is endent upon atrial receptors. The results ined indicate that the renal response to ıl tamponade does not depend upon ıl receptors.

aterials and methods. All experiments were performed on female mongrel dogs averaging 15.6 ± 1 kg (SE) in weight. The chest was opened through a midline sternotomy under aseptic conditions and an atrial pericardial pouch was made by trimming away the pericardium which covered the ventricles and suturing the remaining edge of pericardium to the atrioventricular groove. Thus the atria, but not the ventricles, were enclosed within the pericardial pouch. A catheter was placed into the pouch for the infusion and withdrawal of saline and for recording intrapericardial pressure. Other catheters were implanted in the aorta and superior vena cava, and in one group of dogs a catheter also was placed in the left atrium. Details of the operative procedure and postoperative management have been published (2).

During the thoracotomy, a bilateral partial sympathectomy was performed on all dogs by identifying and cutting the rami communicantes and removing the sympathetic trunk (stellate ganglion through T-5). This level of denervation was chosen because the efferent sympathetic fibers to the heart appear to arise from T-1 through T-4 (4). The more extensive denervation therefore should have eliminated the afferent and efferent impulses that travel in the sympathetic distribution to the heart. For convenience, we will refer to this procedure simply as a "sympathectomy" throughout this paper. In eight animals a vascular occluder with an inflatable cuff was placed around the descending thoracic aorta in order to allow us to control renal arterial pressure. In three of these animals the recurrent laryngeal nerves were cut bilaterally above the origin of the cardiac branches. The dogs were allowed to recover from the operation for at least 1 week.

On the day of the experiment, each dog

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was anesthetized with sodium pentobarbital (30 mg/kg body weight) and both vagus nerves were cut in the midcervical region. In those dogs having an occluder around the aorta, a catheter was inserted into the femoral artery and its tip was advanced to the level of the renal arteries. In all experiments urine flow was elevated above basal levels by the intravenous infusion of saline, and a relatively stable urine flow was established. The total amount of saline infused during prehydration averaged 240 ± 60 ml. Each experiment included a 30-min control period, a 30-min period of atrial tamponade that was produced by infusing saline into the pericardial pouch, and a 30-min recovery period that followed the withdrawal of the saline from the pericardial pouch.

Urine was collected over 10-min intervals through a self-retaining catheter, and a volume of saline equal to the urine volume was infused intravenously at the end of each collection period. The clearance of creatinine was used as an index of glomerular filtration rate. A priming dose of 20 mg of creatinine/kg body weight and a maintenance dose of 0.60 mg/kg $\min^{-1}$ were used. The creatinine was dissolved in 5% dextrose in water and infused intravenously at an average rate of 0.32 ml/min. Blood samples for creatinine analysis were withdrawn at the midpoints of each urine collection period and replaced with an equal volume of 6% dextran in isotonic saline. Creatinine determinations were made with a Technicon autoAnalyzer. Sodium concentrations were determined in duplicate by an IL model 143 flame photometer. Venous and arterial blood pressures were measured with Statham P23Db transducers which were zeroed at the spinous processes of the vertebral column at T4-T5. Pressures were recorded on an Electronics for Medicine DR-8 recorder.

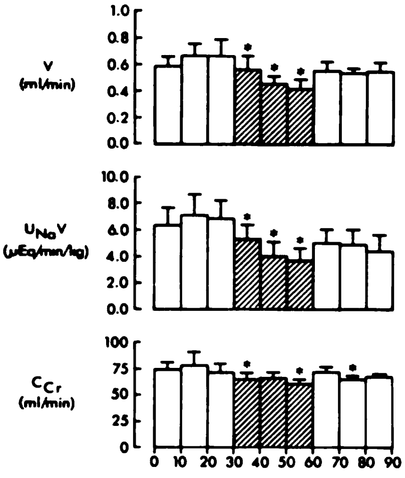
At the end of each experiment, the carotid arteries were occluded bilaterally below the level of the carotid sinuses for 15 sec. This procedure did not produce changes in heart rate in these dogs, thus confirming that the efferent innervation to the heart had been effectively eliminated.

Student's paired *t* analysis (two-tailed) was used to compare data obtained during ˙ 10-min interval of the tamponade and recovery periods with the mean control values. The unpaired *t* test was used to make comparisons between groups of animals. A *P* value of less than 0.05 was considered to be statistically significant.

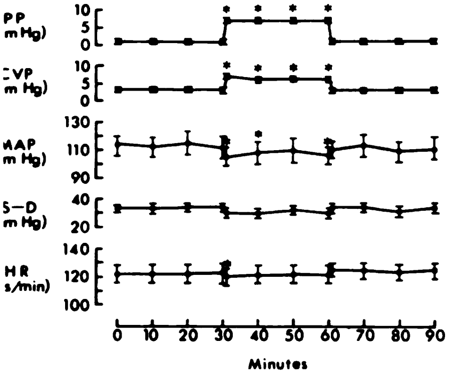
Results. Figure 1 presents a summary of the changes in renal function which occurred during atrial tamponade in six dogs with an acute vagotomy and a chronic sympathectomy. Sodium excretion and urine flow decreased from their respective control values throughout the period of tamponade and returned toward control levels during the recovery period. The relative decreases in sodium excretion and urine flow at the end of tamponade were not different statistically ($P > 0.10$, $P > 0.90$, respectively) from the decreases observed at the end of tamponade in earlier experiments on intact, unanesthetized dogs (1).

The hemodynamic changes produced by atrial tamponade during these experiments are shown in Fig. 2. An increase of approximately 6 mm Hg in intrapericardial pressure increased central venous pressure significantly, a finding that was noted previously (3). Mean arterial pressure was decreased throughout the period of tamponade, a finding in contrast to previous results which demonstrated that mean arterial pressure does not change during atrial tamponade in intact dogs (1, 2). Apparently the extensive autonomic denervation procedures used in the present experiments prevented baroreceptor reflexes from maintaining a constant arterial blood pressure during atrial tamponade. Pulse pressure was not changed significantly from control levels. Heart rate changes were small, but a decrease of two beats/min at the onset of tamponade was significant statistically. This change probably was of no biologic significance, but it may have been elicited by a slight decrease in stretch of sinoatrial pacemaker tissue (5) as a result of the tamponade.

The decrease in arterial pressure which occurred during tamponade in these experiments raised the possibility that a small decrease in renal perfusion pressure could have been directly responsible for the observed decreases in sodium excretion and urine flow. This possibility was examined in experiments conducted on another group of eight dogs that had an inflatable occluder



G. 1. Renal response to atrial tamponade in six athectomized, vagotomized dogs. Values are is ± SE. V, $U_{Na}V$, C_{Cr} denote urine flow, sodium tion, and creatinine clearance, respectively. Con- nd recovery periods are depicted by clear bars and eriod of atrial tamponade by cross-hatched bars. risks indicate means which differ significantly from ol values ($P < 0.05$ or higher levels of signifi-).



G. 2. Hemodynamics recorded in six sympathec- zed, vagotomized dogs. PP, CVP, MAP, S-D, and denote intrapericardial pressure, central venous ure, mean aortic pressure, pulse pressure, and rate, respectively. Other notations as in Fig. 1.

ed around the descending aorta at the
of their thoracotomy. Prior to the col-
on of the control urine samples, the
a was partially occluded to create a pres-
gradient of 15-20 mm Hg between the
ic arch and renal arteries. Subsequently,
ressure within the aortic arch decreased
tly during atrial tamponade the degree of aortic occlusion was reduced in order to maintain the renal artery pressure at control levels or to allow it to rise slightly. The aortic occluder was adjusted in the opposite direction at the end of the tamponade period.

As mentioned previously, the recurrent laryngeal nerves were cut bilaterally in three dogs of this group. The data obtained from these three dogs were combined for analysis with the data from the five other dogs because the decreases in sodium excretion and urine flow during atrial tamponade did not differ ($P > 0.30$ and $P > 0.40$, respectively) between the two groups. The effect of atrial tamponade on renal function in these experiments is summarized in Fig. 3. The magnitude of the decreases in sodium excretion and urine flow in these experiments did not differ significantly ($P > 0.70$ and $P > 0.30$, respectively) from the decreases observed previously in intact, unanesthetized dogs (1).

During tamponade there was an increase in central venous pressure and a decrease in mean aortic arch pressure (Fig. 4). Left atrial pressure and pulse pressure were not statistically different from control levels. Heart rate was not altered significantly dur-

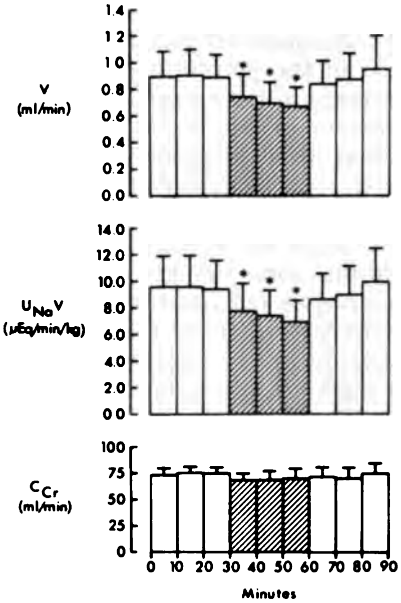


FIG. 3. Renal response in eight sympathectomized, vagotomized dogs in which mean pressure in the renal arteries was maintained constant during atrial tamponade. Notations as in Fig. 1.

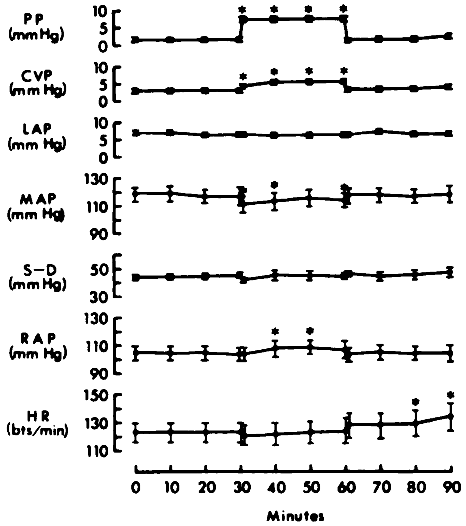


FIG. 4. Hemodynamics recorded in eight sympathectomized, vagotomized dogs in which mean pressure in the renal arteries was maintained constant during atrial tamponade. LAP denotes left atrial pressure, and RAP denotes renal artery pressure. Other notations as in Fig. 2.

ing tamponade but did increase during the latter portion of the recovery period. Pressure at the level of the renal arteries increased slightly, thus the decreases in sodium excretion and urine flow that occurred during tamponade are not attributable to a decrease in the perfusion pressure of the renal arteries in these experiments.

Discussion. These experiments demonstrate that atrial tamponade continues to cause decreases in sodium excretion and urine flow after the known afferent pathways from the heart are severed. Although it is possible that some vestiges of afferent cardiac innervation remained in these dogs, it is highly probable that the majority of fibers were destroyed. The vagus is considered to be the main afferent pathway for impulses from cardiac receptors, and the vagi were divided bilaterally in the midcervical region. In addition, there is considerable evidence indicating that some afferent impulses from the heart reach the central nervous system via the sympathetic rami communicantes (6–8). Therefore all dogs in this study also were sympathectomized from the stellate ganglion through T-5. Although there was no effective way to test for complete efferent denervation of the heart in our studies, we did demonstrate that the efferent pathways were effectively eliminated because bilateral carotid occlusion below the carotid sinuses caused no change in heart rate in these animals. The effectiveness of efferent denervation suggests that afferent cardiac pathways were effectively eliminated also. Finally, since it recently was reported that some afferent impulses from the atria may travel via the laryngeal communicans of the rat (9, 10), we cut the recurrent laryngeal nerves bilaterally in three dogs in order to eliminate this potential pathway for afferent cardiac impulses. Although efferent as well as afferent fibers were eliminated by these procedures, our specific purpose in this study was to eliminate afferent neural pathways from the heart and hence to abolish any reflex effects on renal function which might be initiated by cardiac receptors.

In the experiments in which renal artery pressure was controlled, a direct effect of changes in renal perfusion pressure was ruled out, but it is possible that the reduction in arterial pressure above the occluder may have elicited reflex changes in kidney function which were initiated by carotid sinus baroreceptors. Thus one could argue that the changes in renal function observed during our earlier experiments with intact, conscious animals were mediated at least partly by cardiac reflexes, and that other factors were responsible for the renal response during tamponade in the denervated heart. However, since the magnitude of the antidiuresis and antinatriuresis during atrial tamponade is not altered after cardiac denervation, we favor the conclusion that atrial receptors did not contribute significantly to the renal response elicited by atrial tamponade in our earlier experiments with conscious dogs. We have discussed in detail elsewhere (11) the more general question of whether atrial receptors participate in the physiologic regulation of extracellular fluid volume.

The present experiments did not elucidate the specific mechanisms by which atrial tamponade causes alterations in renal function. The results of this study, however, do imply that the renal excretion of salt and water is quite sensitive to subtle changes in systemic odynamics and that this sensitivity is not ceably altered following rather exten- denervation of the heart.

mmary. Atrial tamponade was used as ethod to reduce atrial transmural pres- in dogs whose afferent pathways from iac receptors were interrupted by sym- ectomy and vagotomy. Under these itions, atrial tamponade caused de- ses in renal sodium excretion and urine which were comparable to those previ- y observed in intact dogs. These obser- ns lead us to conclude that the changes nal function which occur during atrial ponade are not caused by a reflex initi- by atrial receptors. Rather, the altera- s in renal function appear to be attribut- to unidentified changes in systemic he- ynamics that are elicited by atrial tam- de.

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Distribution of Guinea Pig Herpeslike Virus in Brain and Other Tissues of Naturally and Experimentally Infected Animals[1] (39412)

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In experimental infections with different herpesviruses it has been found that the viruses may persist in a latent state for prolonged periods of time without causing apparent disease (1). Latent herpes simplex virus infection of the trigeminal ganglia of mice and rabbits has been studied (2–5) but the distribution of this virus in the central nervous system has not been as extensively explored. Furthermore, even less is known about the neural distribution of other herpesviruses during long-term infection.

A herpeslike virus was isolated from guinea pigs (6), and latent infection of the brain with this virus in experimentally infected animals was studied in our laboratory (7). However, the distribution of this virus in different parts of the brain was not investigated. Because of the importance of latent and persistent viral infections of the nervous system, it was of interest to study the quantitative distribution of virus in the brain and the trigeminal ganglia of guinea pigs naturally and experimentally infected with the guinea pig herpeslike virus (GPHLV). In addition, the distribution of GPHLV in blood, spleen, salivary gland, and other tissues was determined and compared to that of virus in the nervous system.

Materials and methods. Virus stock and cell culture. GPHLV strain Y407, isolated from a naturally infected strain 2 guinea pig was used (6). The virus was passaged in Hartley guinea pig kidney (GPK) cell monolayer cultures four times before use. Virus infectivity titers of the stock virus varied between 10^6 and $10^7 TCID_{50}$/ml. Primary GPK monolayer cultures were prepared from GPHLV-free Hartley guinea pigs, by trypsin dispersion of kidney cells as previously described (8).

Animal source and inoculation. Adult strain 2 and strain 13 guinea pigs were obtained from Dr. C. H. Evans and Dr. D. Rosenstreich, respectively, of the National Institutes of Health. Hartley guinea pigs were purchased from CAMM Research Institute, Wayne, N.J. Hybrid guinea pigs were obtained through cross-breeding of strain 2 and Hartley guinea pigs. GPHLV negative Hartley or F_1 hybrid guinea pigs were observed for any evidence of disease. suspension by the intraperitoneal (ip) or 0.1–0.2 ml by the intracerebral (ic) route. Animals were kept in separate cages and were observed for any evidence of diseases.

Detection of virus. In order to determine onset of natural GPHLV infection, 0.5 ml of heparinized whole blood, obtained from anesthetized animals by heart puncture at monthly intervals, was inoculated onto GPK monolayer cultures. Animals were considered GPHLV positive when virus-induced cytopathic effect (CPE) was observed. Naturally and experimentally infected animals were sacrificed by exsanguination under ether anesthesia. The brain, trigeminal ganglia, spleen, and salivary gland were then removed aseptically. In order to obtain satisfactory samples of the cerebral cortex, diencephalon, brainstem, cerebellum, and trigeminal ganglia, the following procedure was employed. The brainstem was cut at the level of the superior colliculus, and the forebrain and diencephalon were gently raised and lifted out of the cranium. This brain tissue was then sagittally cut and the dience-

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ic area including the basal ganglia, thal- is, and hypothalamus was separated 1 the cerebral cortex. The cerebellum the brainstem were separated by cutting cerebellar peduncles and the middle of medulla oblongata. Care was taken to the trigeminal roots before lifting the nstem out of the cranium. Each trigemi- ganglion was dissected free of the basal inges and separated from the trigeminal . Tissues were handled separately and ɜ trypinisized as described previously Serial 10-fold dilutions of packed cells ›ared from tissues of GPHLV-infected ıals were inoculated into tube cultures ıPK cell monolayers, 3–4 tubes per dilu- . Virus isolation from heparinized whole ›d was performed in a similar manner. ctivity titers of the 10-fold diluted cell ›ensions were determined by the 50% point method (9). In some instances sin-dispersed infected cells were planted eighton tubes as primary cultures. All ıres were examined for the presence of ; and/or virus induced intranuclear in- ıons.

icroscopy of infected tissues. Portions of ɜn, salivary gland, trigeminal ganglion, brain tissue from each animal were ɪ in 10% formalin or Bouin's solution ›wed by staining with H&E for light mi- copy. Small portions of selected tissue ples were also fixed in 3% glutaralde- : and examined by electron microscopy escribed previously (10). For indirect unofluorescence study, blood leuko- ;, and trypsinized spleen cells were fixed with acetone and overlayed with anti-GPHLV serum produced in rabbits, before addition of fluorescein-conjugated anti-rabbit serum (11).

Results. Distribution of GPHLV in brain and trigeminal ganglion of naturally and experimentally infected guinea pigs. Guinea pigs naturally or experimentally infected with GPHLV were observed for periods of up to 15 months. Of the 25 animals studied, 13 naturally and 12 experimentally infected, virus was isolated from the trigeminal ganglia and several parts of the brain of all. Infectivity titers for dispersed cell suspensions of trigeminal ganglia and different parts of the brain are shown in Table I. The range of infectivity titers for the trigeminal and brain tissues was 1.7–2.7 log $TCID_{50}$/ml packed cells, with no significant difference between the naturally infected strain 2, strain 13, or hybrid guinea pigs and the experimentally infected guinea pigs.

Comparison of virus infection in brain, blood, spleen, and other tissues. Since the average infectivity titers of cell suspensions obtained from the different neural tissues did not differ significantly, the titers in the brain and trigeminal ganglia were compared with the titers in other tissues. As shown in Table II, the highest virus titer was found in the spleen regardless of mode of infection, including ic inoculation. The mean titer in spleen was 4.1 log $TCID_{50}$/ml packed cells with lesser titers in kidney, salivary gland, blood, and brain. The average titer in blood was slightly greater than in brain cell suspensions and slightly less than in cell suspen-

'ABLE I. ISOLATION OF GPHLV FROM TRIGEMINAL GANGLIA AND DIFFERENT PARTS OF THE BRAIN OF NATURALLY AND EXPERIMENTALLY INFECTED GUINEA PIGS.

				Average infectivity titers of cell suspensions log $TCID_{50}$/ml packed cells				
uinea pig group	Route of inoculation	Strain of guinea pig	No. animals studied	Tri. gang.[a]	C. Cortex	Dienceph.	Br.St.	Cer.
rally in- ted	None	2	3	1.8	2.2	1.8	1.7	1.7
		13	3	2.2	2.6	2.3	2.3	2.2
		F_1 hybrid[b]	7	1.8	2.2	2.1	2.1	1.9
rimentally :cted	ip	F_1 hybrid	4	2.1	2.2	2.0	2.0	2.1
	ip	Hartley	5	1.9	2.1	1.8	1.8	1.8
	ic	Hartley	3	1.5	1.8	1.8	2.0	1.7

'ri.gang. = trigeminal ganglion; C. cortex = cerebral cortex; Dienceph. = diencephalon; Br. st. = brain Cer. = cerebellum.

train 2 crossed with Hartley.

TABLE II. COMPARISON OF INFECTIVITY TITERS OF CELL SUSPENSIONS OF BRAIN, SPLEEN, AND BLOOD FROM LONG-TERM GPHLV-INFECTED GUINEA PIGS.

Guinea pig group	Route of inoculation	Time postinfection (months)	No. animals studied	Average infectivity titers of cell suspensions log $TCID_{50}$/ml packed cells or whole blood[a]			
				Brain[b]	Blood	Spleen	Salivary gland, kidney, liver
Naturally infected[c]	None	1-5[d]	7	2.0	2.1	4.2	3.2
		7-15	9	1.8	2.3	4.0	3.0
Experimentally infected[e]	ip	4-5	5	1.9	2.7	4.0	Not done
	ip	7-10	5	1.7	2.1	4.2	3.1
	ic	4-5	3	1.7	2.1	4.4	3.0

[a] In guinea pigs in previous experiments (6, 8, 12), GPHLV has also been isolated from lung, uterus, placenta, and thymus of long-term infected animals.

[b] Average of all parts of brain and trigeminal ganglia.

[c] Strain 2, 13, and hybrid F_1 guinea pigs.

[d] For naturally infected animals this was the time after virus was initially detected in blood.

[e] Hartley and hybrid F_1 guinea pigs.

sions from kidney or salivary gland. Occasionally brain tissues of some animals showed titers as high as those in whole blood. Other tissues, including lung and liver showed titers similar to those in kidney and salivary gland.

In naturally infected guinea pigs the presence of virus infection was initially determined by isolation of virus from whole blood. These animals were sacrificed 1-15 months after first evidence of virus infection, and virus was reisolated from the blood of all animals at time of sacrifice. None of the GPHLV-infected animals showed clinical evidence of nervous system or other disease during the period of observation.

Microscopy of infected tissues. Light microscopy of infected tissues from both naturally and experimentally infected animals did not show intranuclear inclusions or other pathology. Several attempts to visualize viral antigens on leukocytes and spleen cells from GPHLV-infected guinea pigs using indirect fluorescent antibody techniques were negative. Following electron microscopic examination, virions were not found in tissues from virus positive animals.

Discussion. The present study showed that in natural or experimental infection of guinea pigs with GPHLV, virus could be isolated in similar quantity from the trigeminal ganglion and several parts of the brain. Virus was present in greater quantity, however, in blood, spleen, kidney, and salivary gland than in the brain. In previous studies it was found that the GPHLV isolated from the blood of infected guinea pigs was associated with the leukocytes (10, 12). Latent infection of GPHLV in guinea pigs, therefore, may be somewhat similar to EB herpesvirus infection in humans. The association of virus with circulating leukocytes could explain the similarity between titers in the trigeminal ganglia and in the different parts of the brain sampled, without localization to any particular tissue. If virus isolated from the neural tissues was indeed due to contamination by leukocytes, it would indicate the importance of testing for the presence of viremia. Thus, determination of viremia, as well as study of the distribution of virus in the nervous system, would be necessary prior to demonstrating the localization of a virus to a particular part of the nervous system.

Infection with GPHLV is in keeping with some of the concepts of latent virus infection. Long-term infected guinea pigs are apparently normal, and examination of infected tissues by light and electron microscopy has not revealed evidence of histopathology or of viral inclusions or virions (8, 10). Similarly, infected leukocytes or spleen cells from guinea pigs have not revealed evidence of viral antigens following immunofluorescence testing. Thus, as in the present experiment, once infection has been initiated, the animals are chronically but subclinically infected. Demonstration of the presence of virus in infected tissues has been accomplished only by primary cultivation, or cocultivation with susceptible cells of intact infected tissue cells, but not homogenates of infected tissues (6). In contrast, infection with guinea pig cytomegalovirus (GPCMV), another herpesvirus of guinea pigs, is quite different from infection of guinea pigs with GPHLV. In a separate study we found that intranuclear inclusions and virions were observed in tissues of guinea pigs infected with GPCMV, and infectious virus was readily isolated only from salivary gland (13).

Summary. Distribution of GPHLV in the trigeminal ganglion, brain, and other tissues of guinea pigs was studied. Virus was found in suspensions of all tissue cells from naturally infected guinea pigs, as well as animals inoculated ip or ic, up to 15 months after initiation of infection. Evidence of virus infection was detected by cultivation of cells or by cocultivation of infected cells with susceptible cell cultures, but not by other methods including immunofluorescence and electron microscopy. Quantitative determinations of virus in the trigeminal ganglia and different parts of the brain showed infectivity titers to be similar in these tissues. Since GPHLV was also found in blood and in high titer in spleen, and in other tissues tested, isolation of virus from neural tissues may have been due to contamination with infected blood leukocytes.

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Inhibition of HCG-Induced Ovarian and Uterine Weight Augmentation in the Immature Rat by Analogs of GnRH (39413)

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It is firmly established that human chorionic gonadotropin (HCG) increases the sensitivity of the immature rat ovary to exogenous follicle stimulating hormone (FSH) and that the relationship between FSH and ovarian weight as shown by Steelman and Pohley (1) is linear. The reliance of ovarian weight changes following augmentation of FSH by HCG has gained widespread use as a quantitative bioassay for the measurement of FSH in various preparations (1, 2).

In collaboration with Takeda Industries, Ltd., we demonstrated in a number of studies that several analogs of the synthetic decapeptide, *p*-Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH$_2$ (GnRH), have intensified ovulation-inducing and gonadotropin-releasing activity. For example, replacement of the C-terminal glycine amide of GnRH with various alkylamines resulted in a three- to fivefold increase in ovulation induction and *in vitro* LH and FSH release (3–5). At about the same time, Monahan *et al.* (6) reported that [D-Ala^6]-GnRH was also more active than GnRH. Subsequently, we reported that analogs containing both a C-terminal ethylamide and position 6 D-amino acid modification possessed intense biological activity (7, 8). One of these analogs, [D-Leu^6, des-GlyNH$_2$^{10}, Pro-ethylamide^9]-GnRH (I), was at least 30 to 100 times more active than GnRH in causing gonadotropin release or ovulation in either the mature female rat, ewe, or estrous rabbit (9).

On the basis of these observations we designed experiments to determine if I released sufficient FSH *in vivo* to allow for augmentation by HCG in the Steelman-Pohley rat. To our surprise, large doses of I inhibited the usual ovariogenic and uterotropic effects of HCG. Thus, this report examines in detail the inhibitory effect of I on HCG augmentation and describes preliminary findings with other analogs of GnRH.

Methods. The synthesis, purification, characterization, and biological properties of the peptides studied in these experiments were reported previously (3, 7, 8). Sprague-Dawley female rats were obtained at 21 days of age (Sprague-Dawley Co., Madison, Wis.) and maintained in colony cages in quarters with controlled temperature (26 ± 1°) and lighting (lights on 5 AM to 7 PM). Tap water and ground laboratory chow were available *ad libitum*. Hypophysectomized rats (Altech Laboratory, Madison, Wis.) were given access to 10% glucose-saline in place of tap water. Injections were initiated at 22 days of age according to the procedures of Steelman and Pohley (1). Each rat was injected sc at 8 AM and 4 PM for 3 consecutive days, unless indicated otherwise. The rats were killed with an overdose of ether at 25 days of age and the ovaries and uteri removed, cleaned, and weighed to the nearest 0.2 mg on a Roller-Smith balance. One group of animals was injected for various periods of time and checked twice daily for vaginal opening. All solutions were made with buffered saline (10) and stored at 4°. In most cases, HCG[1] and the peptide or the gonadotropin were administered as a single injection. The data were analyzed by analysis of variance and differences between treatments compared using orthoganol contrasts or Duncan's Multiple Range Test (11).

Results. HCG increased both ovarian and uterine weight of the immature rat and augmented the effect of FSH on ovarian weight (Table I: Expt. 1). The relatively large dose of 5 μg of I administered during the 3-day test period did not affect ovarian or uterine

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[1] Antuitrin "S," Parke-Davis, Detroit, Michigan.

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TABLE I. EFFECT OF [D-LEU^6, des-GLYNH$_2$^{10}, PRO-ETHYLAMIDE^9]-GnRH (I), GnRH, LH, AND FSH ON MEAN OVARIAN AND UTERINE WEIGHT IN HCG-AUGMENTED IMMATURE RATS.

Group[a]	Dose of peptide (µg/rat)	Ovarian weight (mg ± SE)	Uterine weight (mg ± SE)
Experiment 1			
Buffer	–	13.8 ± 0.6	28.8 ± 2.0
HCG	–	41.4 ± 0.3	116 ± 4.4
HCG + FSH (90 µg)[b]	–	120 ± 21	129 ± 7.4
I	5	10.6 ± 0.2	26.8 ± 2.0
HCG + I	0.008	42.6 ± 2.3	109 ± 8.0
	0.04	41.6 ± 3.5	100 ± 4.5
	0.2	27.5 ± 0.6	91.8 ± 8.7
	1	17.2 ± 1.6	41.6 ± 5.6
	5	12.5 ± 1.3	31.8 ± 2.0
HCG + GnRH	500	20.2 ± 1.6	96.4 ± 8.5
	2500	15.8 ± 0.9	38.6 ± 2.8
Experiment 2			
HCG	–	37.3 ± 2.3	110 ± 4.4
HCG + I	5	11.4 ± 1.0	28.0 ± 2.2
HCG + LH (12.5 µg)	–	34.2 ± 1.0	114 ± 4.9
HCG + FSH (90 µg)	–	82.2 ± 13	118 ± 2.3
HCG + LH + I	5	10.9 ± 0.9	26.7 ± 1.2
HCG + FSH + I	5	10.6 ± 0.9	29.4 ± 3.2
Experiment 3			
HCG	–	34.1 ± 2.6	
I	5	10.2 ± 0.9	
HCG + I (−2 hr)[c]	5	10.8 ± 0.4	
(−4 hr)	5	11.8 ± 0.4	
(+2 hr)	5	11.9 ± 1.4	
(+4 hr)	5	10.8 ± 0.7	
HCG (AM only)	–	37.2 ± 2.2	
HCG (AM) + I (PM)	5	22.8 ± 2.0	
I (AM) + HCG (PM)	5	16.8 ± 1.0	
HCG (Days 2 and 3)	–	20.6 ± 1.1	
I (Day 1) + HCG (Days 2 and 3)	5	20.7 ± 1.3	

[a] Five rats per group. Each rat received 50 IU of HCG. All animals were injected twice a day (22–24 days of age) unless indicated otherwise.

[b] Numbers in parenthesis indicate total dose per rat.

[c] Time interval before (+) or after (−) HCG injection.

weight when rats did not receive HCG along with the peptide. However, dose levels of 0.2 to 5 µg of I effected a dose response inhibition ($P < 0.01$) of the increases in ovarian and uterine weight induced by HCG. At the 5-µg dose of I, ovarian and uterine weights were not significantly different from buffer controls. GnRH, injected at much higher levels, was also inhibitory.

The inhibitory effect of I also occurred when rats received either exogenous LH in addition to HCG, or when FSH was augmented by HCG (Expt. 2). In Expt. 3, when I was injected either 2 or 4 hr before or after HCG, ovarian augmentation was inhibited to the same extent as when both hormones were administered together. Injecting I at 8 AM and HCG at 4 PM resulted in a greater inhibition of ovarian augmentation ($P < 0.05$) than when the sequence was reversed. Moreover, when the total dose of I was given on Day 1 and HCG on Days 2 and 3 of the treatment period, inhibition of ovarian weight augmentation did not occur.

Increasing the 3-day augmenting dose of HCG from 50 to 500 IU did not overcome the inhibition by I of ovarian weight increase (Table II). Moreover, graded levels of exogenous LH (10, 20, and 40 µg) did not affect ovarian weights of control or HCG-treated rats. Hypophysectomy did not alter the inhibitory effect of I as relative changes in ovarian weight were similar to those in intacts (Table III).

TABLE II. EFFECT OF HCG, LH, AND [D-LEU^6, des-Gly NH$_2$^{10}, PRO-ETHYLAMIDE^9]-GnRH (I) ON OVARIAN WEIGHT (MEAN ± SE) IN IMMATURE RATS.[a]

		Dose of HCG (IU/rat)		
		0	50	500
I (μg/rat)	0	11.7 ± 1.3	38.4 ± 4.1	34.6 ± 2.9
	0.32		19.9 ± 0.9	21.5 ± 1.9
	0.80		16.2 ± 1.7	17.4 ± 1.5
	2.0		14.8 ± 1.4	13.6 ± 0.8
	5.0	10.3 ± 0.8	11.9 ± 0.7	11.0 ± 0.6
LH[b] (μg/rat)	0	13.8 ± 0.8	37.3 ± 2.2	
	10	13.4 ± 0.3	41.2 ± 2.2	
	20	13.6 ± 0.4	42.0 ± 3.9	
	40	14.8 ± 0.8	36.1 ± 4.7	

[a] Injected twice daily: 22–24 days of age.
[b] Potency of 1.6 × NIH-LH-S1.

TABLE III. MEAN OVARIAN WEIGHTS IN INTACT AND HYPOPHYSECTOMIZED IMMATURE RATS AFTER GONADOTROPIN AND [D-Leu^6, des-Gly NH$_2$^{10}, PRO-ETHYLAMIDE^9]-GnRH (I) TREATMENT.

	Intact		Hypophysectomized	
Group[a]	No. rats	Ovarian weight (mg ± SE)	No. rats	Ovarian weight (mg ± SE)
Control	15	15.0 ± 0.5	18	5.5 ± 0.3
HCG (50 IU)	15	51.6 ± 3.2	11	12.5 ± 1.4
HCG + FSH (180/μg)	5	121.0 ± 11.0	6	37.2 ± 5.4
HCG + I (5 μg)	15	15.2 ± 1.0	14	4.7 ± 0.3

[a] Injected twice daily: 22–24 days of age.

Table IV shows that both HCG and HCG + I injected on Days 22–24 of age significantly ($P < 0.01$) advanced the age at vaginal opening (29 vs 40 days for both controls and I alone, respectively). Twice a day administration of 0.05 or 0.5 μg of I on Days 22–31 of age also resulted in a significant ($P < 0.01$) reduction in age at vaginal opening (36 vs 40 days for controls). However, continuing the twice daily injections until puberty effected a 5-day delay in vaginal opening at 0.5 μg ($P < 0.01$), but not at the 0.05-μg dose.

That inhibition of ovarian augmentation in the HCG model is not limited to one analog of GnRH is shown in Table V. For example, [D-Ala^6, des-GlyNH$_2$^{10}, Pro-ethylamide^9]-GnRH (analog IV), another potent agonist of GnRH, was as active as I in inhibiting HCG augmentation. Two less active agonists of GnRH, [des-GlyNH$_2$^{10}, Pro-ethylamide^9] (II) and [D-Leu^6] (III), also inhibited HCG augmentation but to a lesser extent than either I or IV. In contrast, the des-His^2 analog of [D-Leu^6]-GnRH (analog V) increased ovarian weight at 1 and 5 μg ($P < 0.05$) as did the des-His^2 analog of I (analog VI) at the 0.2-μg dose level.

Discussion. In addition to an intense gonadotropin agonist activity, [D-Leu^6, des-GlyNH$_2$^{10}, Pro-ethylamide^9]-GnRH (I) exerts a pronounced inhibition of ovarian and uterine augmentation of HCG in the immature rat, as demonstrated in the present experiments. This surprising biological effect was dose-related. Moreover, the peptide effectively inhibited ovarian and uterine weight increase when injected either with or at a site separate from HCG, or when administered up to 4 hr before or after HCG. An interval of 8 to 16 hr between injection of I and HCG, regardless of which was given first, resulted in a lesser inhibition of HCG augmentation than at reduced intervals. Moreover, a level of HCG 10 times that normally used in the Steelman–Pohley bioassay model for FSH did not reduce the inhibitory effect of I. The increase in ovarian weight after HCG augmentation of exogenous FSH was also inhibited by the analog.

The mechanism whereby I inhibited ovar-

l uterine augmentation of HCG in the
t studies remains obscure. Johnson
allampati (12) observed that the im-
: 23-day-old rat released large quanti-
both FSH and LH when injected ip
relatively low level of GnRH (40 ng).
'er, in our experiments exogenous
.5-64 μg of activity, per rat) did not
ɔvarian weight either in the presence
ɜnce of HCG, suggesting that the in-
y effect of I on HCG augmentation
e extrapituitary, possibly uterine or
ı. In support of this supposition, we
ed that I also inhibited HCG augmen-
in the hypophysectomized rat. It is
y that sufficient residual gonadotro-
ɔresent in the acutely hypophysecto-
rat which accounts for a response
to that observed with the intact ani-
loreover, we recently observed (un-
ıed) that I effectively inhibited ovar-
ıght augmentation of HCG in mature
10 weeks after hypophysectomy.
ɜrtheless, the inhibition of HCG-in-
ovarian and uterine augmentation by
)ther analogs of GnRH was, in gen-
roportional to their intrinsic LH and
leasing activities reported previously
In the immature male rat, 0.5 μg of
ic GnRH administered once daily for
ks significantly decreased accessory
organ weight (13). These workers
ed evidence that the response was
to altered steroid biosynthesis. Re-
Banik and Givner (14) reported that
IV (our designation) advanced ovu-
in the diestrous rat and prevented mating behavior and pregnancy during the ensuing expected estrus period. It was suggested that this antifertility effect was the result of a change in ovarian steroidogenesis as measured by vaginal cytology. In our studies vaginal opening was delayed 5 days when 0.5 μg of I was administered twice a day from 22 days of age, but advanced 4 days if the peptide injections terminated at 32 days of age. In another series of experiments (Johnson *et al.*, in press), chronic administration of I inhibited normal ovarian growth, maturation, and vaginal opening in the immature rat, and prevented normal cycling and caused atrophy of the ovaries and uterus in mature animals. These results together with the work of others suggest an effect of large doses of I on ovarian steroidogenesis, but do not indicate if the mechanism is direct.

E IV. INFLUENCE OF [D-Leu^6, des-GlyNH$_2^{10}$,
HYLAMIDE^9]-GnRH (I) AND HCG ON AGE AT
GINAL OPENING IN THE IMMATURE RAT.

ɔup[a]	Dose of I per injection (μg/rat)	Days of age injected	Age at vaginal opening (mean ± SE)
	–	22-24	40.0 ± 0.4
0 IU)[b]	–	22-24	29.2 ± 0.4
I	0.83	22-24	29.4 ± 0.4
	0.83	22-24	39.6 ± 0.3
	0.05	22-31	36.2 ± 0.4
	0.5	22-31	36.2 ± 0.5
	0.05	22-open	40.2 ± 1.2
	0.5	22-open	45.0 ± 2.0

: rats per group injected daily at 8 AM and 4

al dose per rat.

TABLE V. EFFECT OF VARIOUS ANALOGS OF GnRH ON HCG-AUGMENTATION OF OVARIAN WEIGHT (mg ± SE) IN THE IMMATURE RAT.

	Total dose of peptide (μg/rat)[a]		
Peptide	0.2	1.0	5.0
I	27.4 ± 1.3	18.2 ± 1.4	11.9 ± 0.3
II	38.4 ± 3.0	30.2 ± 1.8	22.2 ± 1.6
III	32.0 ± 1.3	24.6 ± 2.4	17.6 ± 2.0
IV	25.9 ± 2.4	18.3 ± 1.8	13.2 ± 1.5
V	34.8 ± 2.7	42.0 ± 3.0	47.2 ± 4.2
VI	42.0 ± 3.5	39.7 ± 3.6	37.8 ± 2.3
	100 μg	500 μg	
GnRH	22.4 ± 0.93	16.1 ± 1.1	

[a] Five rats per group injected at 8 AM and 4 PM for 3 consecutive days. Ovarian weight of HCG controls (50 IU/rat) was 33.3 ± 2.0 mg.

Peptide I: [D-Leu^6, des-GlyNH$_2^{10}$, Pro-ethylamide^9]-GnRH; Peptide II: [des-GlyNH$_2^{10}$, Pro-ethylamide^9]-GnRH; Peptide III: [D-Leu^6]-GnRH; Peptide IV: [D-Ala^6, des-GlyNH$_2^{10}$, Pro-ethylamide^9]-GnRH; Peptide V: [des-His^2, D-Leu^6]-GnRH; Peptide VI: [des-His^2, D-Leu^6, des-GlyNH$_2^{10}$, Pro-ethylamide^9]-GnRH.

In conclusion, we have demonstrated a pronounced negative relationship between the dose of potent agonists of GnRH and the ability of HCG to augment ovarian and uterine weight in the immature rat. It remains to be demonstrated if this effect involves other endocrine organs, such as the pineal or adrenal, or if the analogs act directly on ovarian or uterine tissue.

Summary. Experiments were conducted to study the inhibitory effect of large doses of a few analogs of GnRH on ovarian and

uterine weight in the immature rat. In most experiments the 21-day-old HCG-primed animal was used. Extensive study of [D-Leu^6, des-GlyNH$_2$^{10}, Pro-ethylamide^9]-GnRH (I) indicated that ovarian weight increase induced by HCG was inhibited in both the intact and hypophysectomized rat. Increasing the augmenting dose of HCG from 50 to 500 IU in the intact animal did not overcome the inhibitory effect of I. Furthermore, twice-a-day injections of I into normal immature rats from 22 to 31 days of age significantly advanced puberty; however, when injections were continued until puberty, vaginal opening was significantly delayed. In general, the inhibitory effect of other analogs of GnRH in the HCG model was proportional to their intrinsic LH and FSH releasing activities. These data constitute direct evidence that large doses of the intensely active analogs of GnRH have inhibitory effects on the ovary, uterus, and reproductive function in the rat.

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Protective Effect of Clofibrate Against *S. pneumoniae* Infection in Rats[1] (39414)

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Pneumococcal sepsis is associated with decreased plasma albumin concentration and increased acute phase globulin synthesis (1–3) even in protein deficient or starved animals, implying that altered plasma protein metabolism is a fundamental host response to infection. Hepatic peroxisome content is decreased during pneumococcal sepsis; and it has been suggested that the increased acute phase serum protein synthesis occurs at the expense of peroxisomal protein synthesis (4). Increased cholesterolgenesis (5) and triglyceride synthesis (6) have also been reported to occur in pneumococcal infection. The inverse relationship between hepatic peroxisomal content and lipid metabolism during infection may be more than coincidence in that peroxisomes are thought to control intracellular cholesterol level by regulating its degradation (7) and drugs which lower serum cholesterol and triglyceride concentration induce hepatic peroxisomal proliferation (8). Clofibrate (ethyl-*p*-chlorophenoxyisobutyrate), an antihyperlipidemic agent produces diametrically opposite changes in metabolism compared to pneumococcal sepsis, i.e., increased plasma albumin concentration and decreased acute phase globulin levels (9), increased catalase synthesis (10), and decreased plasma cholesterol and triglyceride concentrations (11). It was therefore of interest to ascertain whether clofibrate would mute or enhance the severity of pneumococcal sepsis in rats.

Materials and methods. Fisher Dunning rats (Microbiological Associates, Walkersville, Md.) weighing 150–200 g were housed in a room maintained at 25–28° and were fed *ad libitum* on commerical rat chow (Purina). Clofibrate, provided by Dr. George Brice (Ayerst Laboratories, N.Y.) was dissolved in 95% ethanol and added to ground commercial chow on a wt/wt basis. The drug-treated chow was allowed to air-dry to remove the ethanol before being fed to the rats. Virulent *Streptococcus pneumoniae* type 1, strain 5, served as the infectious agent. Infection was initiated by the sc injection of the requisite number of organisms in 0.1 ml of saline. Bacteremia was quantitated by plating 0.1 ml of serial 10-fold dilutions of whole blood onto blood agar plates and counting the resulting colonies after 24 hr of incubation at 37°. Plasma chlorophenoxyisobutyrate (CPIB) content was determined by the spectrophotometric method of Barret and Thorpe (11). Clofibric acid for standardization was obtained from Dr. Dvornik (Ayerst Laboratories, Montreal, Canada).

For carbon clearance studies rats were lightly anesthetized with halothane and then injected via the dorsal penile vein with a suspension of colloidal carbon (Pelikan ink, Gunther Wagner) at a dose of 8 mg/100 g body wt. At various intervals 25-μl blood samples were obtained from the tail vein, mixed with 2.0 ml of 0.1% Na_2CO_3, and the optical density measured at 620 nm. The clearance rate (K) was calculated according to Gorelkin and Jahrling from the formula $K = \log C_1 - \log C_2/t_2 - t_1$. C_1 and C_2 are colloidal carbon concentrations in mg/100 ml of blood, $C = \mathrm{O.D.}_{620} \times 48.5$, and t_1 and t_2 are times of sampling in minutes (12).

Results. Rats were fed a normal diet or one containing either 0.25 or 1.25% clofibrate (wt/wt) for 1 week before challenge with 3×10^2 *S. pneumoniae* injected sc. All untreated rats were dead in 7 days, while 5 of 20 fed the 0.25% diet and 17 of 20 fed the 1.25% diet survived (Fig. 1). Clofibrate

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[1] In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association of Accreditation of Laboratory Animal Care.

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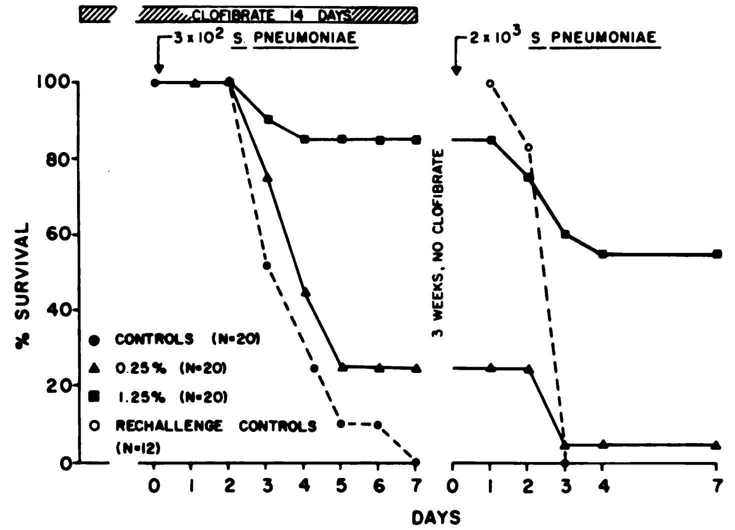


FIG. 1. Effect of dietary clofibrate on survival in rats inoculated with *S. pneumoniae*.

was removed from the diet 7 days postinfection and 3 weeks later the survivors plus a new set of controls were challenged with 2 × 10^3 organisms of the same strain of *S. pneumoniae*. All control rats died within 3 days, only 1 of 5 of those rats which originally had been on the 0.25% diet survived while 11 of 17 of those that had been on the 1.25% diet survived.

Since clofibrate protected some rats against death due to pneumococcal sepsis, another set of control and drug-treated (1.25%) rats were inoculated sc with 10^3 *S. pneumoniae* and animals were sacrificed at 24-hr intervals to determine bacteremia. Pretreatment with clofibrate caused a significant decrease in bacteremia at 48 hr as well as a decrease in the number of animals with bacteremia. Although both the number of bacteria/ml blood and the number of rats with positive cultures were equally low in the drug-treated group at 72 hr, no statistically significant difference was measurable owing both to the paucity of survivors in the untreated group and the large standard deviation in the drug-treated groups (Table I).

Since clofibrate not only protected rats against *S. pneumoniae* infection but also decreased the incidence and extent of bacteremia, plasma from rats fed various levels of dietary clofibrate were tested to determine if the drug had a bactericidal or bacteriostatic activity. Nine-hundred *S. pneumoniae* were incubated with plasma; samples were withdrawn for plating at 30 min and 3 hr. There was no change in bacterial count after 30-min incubation, indicating that the active form of clofibrate in the plasma (CPIB) does not have bactericidal activity. The comparable growth evident at 3 hr in all instances indicated that CPIB does not have a bacteriostatic effect (Table II).

TABLE I. BACTEREMIA[a] AFTER SUBCUTANEOUS INJECTION OF 10^3 *S. Pneumoniae*.

	Control		1.25% Clofibrate	
Time (hr)	Log titer/ml	No. positive/total	Log titer/ml	No. positive/total
24	0	0/5	0	0/5
48	5.2	5/5	3.8[b]	3/5
72	5.7	3/3	3.9	3/5
96	No survivors		0	0/3

[a] Bacteremia determined in duplicate.

[b] $P < 0.01$ vs. control.

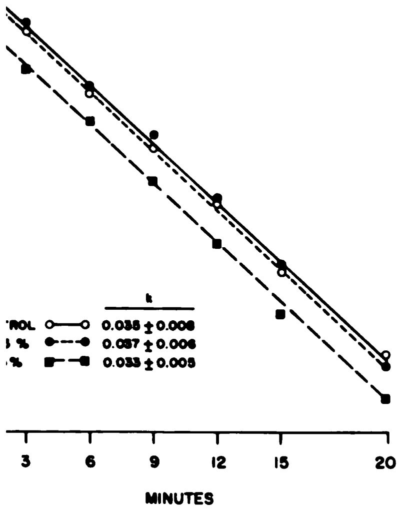
Since clofibrate at the levels used in this study induces hepatomegaly, it is possible that clofibrate might induce a proliferation of the reticuloendothelial system as well and thus enhance the phagocytosis of the *S. pneumoniae*. As can be seen in Fig. 2 the rate of clearance of colloidal carbon from the blood of rats fed clofibrate for 10 days was the same as that of control rats. The *K* value for control rats was 0.035 ± 0.008 (mean ± SD), a value similar to that previously reported for normal rats (13).

Discussion. Clofibrate added to the diet

E II. PLASMA CPIB CONTENT AND ANTIBACTERIAL ACTIVITY.

Plasma CPIB µg/ml[a]		Organisms/ml + SE[b]	
Mean ± SE	Range	30 min	3 hr
–	–	1095 ±140	3148 ±472
33 ±5	(28-44)	1095 ±260	2856 ±723
109 ±8	(86-174)	1035 ±95	2592 ±476
246 ±31	(114-372)	1165 ±250	2856 ±728

Nine-tenths milliliter of plasma was mixed
of saline containing 900 *S. pneumoniae* and
37°. Duplicate 0.2-ml samples were taken
d 3 hr; bacteria were quantitated by plating
ial 10-fold dilutions onto blood agar plates
the resulting colonies after 24 hr of incu-
°.



Carbon clearance in normal rats and in
rious amounts of clofibrate in the diet.

k prior to infection with *S. pneu-*
rotects some rats from what would
be a uniformly lethal infection.
ge of the survivors with the same
S. pneumoniae in the absence of
indicates that many of the rats
had been exposed to sufficient
mass to induce immunity. Despite
omegaly induced by clofibrate, it is
hat the drug diminishes bacteremia
by enhancing the phagocytosis of the bacteria since carbon clearance, an indicator of phagocytic activity, is unaltered in drug-treated rats. Nor is there any consistent, significant effect of the drug on total leukocyte or differential count which might account for the protection afforded. It is apparent that although plasma containing the active form of the drug is neither bactericidal nor bacteriostatic *in vitro*, clofibrate still decreases the incidence and extent of bacteremia *in vivo*. Although clofibrate does not exhibit bactericidal or bacteriostatic action *in vitro*, it is nonetheless still possible that the drug may deleteriously affect the metabolism of bacteria which are growing within an animal or restrict the availability of substrate, thereby lessening microorganism replication. Grossberg *et al.* (14) have proposed that clofibrate decreases viral replication in some instances by limiting the supply of lipids necessary for virus membrane formation. Weinberg (15) has suggested that the decrease in plasma iron which occurs in numerous infectious illnesses may, at least in some instances, be beneficial to the host in restricting microbial growth. Clofibrate does, in fact, occasion a decrease in plasma iron concentration and in that of zinc, iron, and copper (unpublished data), as well as decreasing plasma cholesterol and triglyceride concentrations (11) and altering serum protein patterns (9).

The precise mechanism of action of clofibrate's protection against pneumococcal sepsis in the rat, as well as the implications of this protection as regarding the essentially of certain of the host's responses to infection (1-6), remains to be determined.

Summary. Clofibrate, nominally an antihyperlipemic agent, added to the diet for a week prior to the inoculation of rats with *S. pneumoniae* protected some of the rats from what would normally be a uniformly lethal infection. Plasma from clofibrate-treated rats was neither bactericidal nor bacteriostatic, yet drug-treated rats displayed significantly diminished incidence and extent of bacteremia. Clofibrate does not alter carbon clearance, thus it is unlikely that clofibrate affords protection through enhanced reticuloendothelial removal of microorganisms. The mechanism and implications of clofibrate protection remain to be determined.

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GS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 441–448 (1976)

The Effect of Stress on Serum Prolactin in the Female Rat[1, 2] (39415)

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effect of stress on endocrine control nisms is receiving increased attention. al investigators have demonstrated arious stressors can increase serum tin levels in both male and female rats than on the afternoon of proestrus (1- e elevated serum prolactin levels on ternoon of proestrus were not affected least one commonly used stress, ether (2, 7). Stress has been associated with ed reproductive performance in many s. These effects of stress on reproduc- ave included blockage of sexual cy- lecreased implantation rates, and in- d embryonic mortality (8–11). Al- h stress is usually associated with in- d serum prolactin concentrations, re- lata from our laboratory showed de- d serum prolactin and inhibition of ion in female rats given restraint on the afternoon of proestrus (12). In n a recent study has indicated that stress can inhibit prolactin release in the afternoon of proestrus (13). The t study was undertaken to determine fect of stressors of varying intensities lactin secretion in rats in a variety of logical states, including cycling, cas- , pregnancy, and lactation.

erial and methods. Young adult (4–8 s) female Long-Evans (Blue-Spruce , Almont, N.Y.) were used in these s. The rats were housed in a tempera- ntrolled (72 ± 2°F) and artificially (12-hr light cycle) room and main- on Wayne Lab-Blox and water sup- *d libitum.* Daily vaginal smears were Only rats exhibiting at least two normal estrous cycles before experimentation were included in the study.

All blood samples were collected by orbital sinus puncture. The volume of each blood sample was about 1 ml. The adequacy of orbital sinus bleedings in the determination of "control or nonstressed" serum prolactin concentrations was studied by comparing serum prolactin in blood samples taken by orbital sinus and by rapid decapitation techniques. At 4 PM on the days of estrus, proestrus, and diestrus Day 2, individual rats were rapidly transferred from their cages directly into ether chambers and an orbital sinus blood sample was taken between 40 and 60 sec after initial animal disturbance (designated as a 1-min blood sample). The rats were returned to their cages. At 4 PM on the third estrous cycle after orbital sinus sampling, the same rats at the same stages of their estrous cycles were individually removed from their cages, transported to an adjacent room and a blood sample was taken by decapitation within 15 sec of animal disturbance.

Separate groups of rats at proestrous, estrous, and diestrous 2 stages of their ovarian cycles were subjected to one of three types of stressors: ether vapor, ether vapor plus serial bleeding, and restraint. Rats exposed to ether vapor stress were removed from their cages and placed in ether chambers. Following anesthesia, they were removed from the chambers and allowed to regain consciousness. The same rats were then reintroduced into the ether chambers at 8, 15, and 25 min after removing them from their cages. A single blood sample was taken by orbital sinus puncture after the 25-min anesthesia. Control prolactin levels for this experiment were measured in orbital sinus blood taken within 1 min of animal disturbance from separate groups of rats. The second stress, ether vapor plus serial bleeding, utilized the same anesthesia scheme as described for ether vapor stress, except serial

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served.

blood samples were taken by orbital sinus puncture at each anesthesia interval (1, 8, 15, and 25 min). Prolactin concentrations in the 1-min blood sample was designated as the controls for this experiment. The third stressor employed was restraint. Groups of rats were removed from their cages, placed in ether chambers and an orbital sinus blood sample taken within 1 min of initial disturbance. The rats were then restrained in the supine position for 2 hr. At the end of the 2-hr restraint, rats were released, placed back into ether chambers, and a second blood sample taken within 1 min from the end of the restraint period. Serum prolactin levels in the prerestraint blood samples were considered as controls for this experiment.

The effect of chronic stress on serum prolactin was determined by subjecting a group of rats on the first day of diestrus to 20 consecutive days of 2-hr restraint stress. Serum prolactin levels were measured in serial blood samples taken within 1 min of animal disturbance in their cages on Days 1, 9, and 19. These prestress prolactin levels were designated as control hormone levels. The effect of the chronic restraint on serum prolactin was measured in additional blood samples taken within 1 min of the end of the restraint period on Days 1, 5, 10, 15, and 20 of the treatment regime.

Another experiment measured the effect of 2-hr restraint stress on serum prolactin on the first day of pregnancy. Separate groups of rats received 2-hr restraint stress treatments from 12-2 PM or 3-5 PM on the day sperm were detected in the vaginal smear (Day 1 of pregnancy). Serum prolactin was measured in serial blood samples taken at 12, 2, 3:30, and 5 PM from the 12-2 PM stress groups, and at 3, 5, 6:30, and 8 PM from the 3-5 PM stress group. Control prolactin levels were measured in serial blood samples taken by the rapid sampling technique at the same time intervals from similar groups of rats that did not receive the restraint stress treatment.

The final experiment included in this study considered the effect of serial bleeding and restraint stressors on prolactin secretion in lactating rats. Rats included in this study were in the fourth to tenth day of lactation and were observed to be over their pups in the interval before experimentation. For the serial bleeding study, rats were removed from their cages, placed in ether chambers. and bled at 1, 8, 15, and 25 min following first disturbance. For the restraint portion of the experiment, rats were restrained for 2 hr after collection of the 1-min blood sample with a second blood sample taken within 1 min of the end of the restraint period.

Serum prolactin was measured in duplicate by a standard double-antibody radioimmunoassay procedure (14). NIAMD-Rat Prolactin, RP-1 was used as the reference standard. Differences between groups were analyzed by Student's *t* test and a probability of 0.05 was considered as significant.

Results. Comparison of control or nonstressed serum prolactin levels measured in the same rats by two experimental techniques, rapid orbital sinus puncture and decapitation is shown in Fig. 1. No differences were recorded in serum prolactin concentrations measured by these techniques at any stage of the estrous cycle.

Figure 2 illustrates the effect of exposure to a mild stress, ether vapor, in rats at proestrous, estrous, and diestrous 2 stages of their ovarian cycle. Serial exposure to ether vapor over the 25-min experimental interval (the rats were anesthetized at 1, 8, 15, and 25 min after removing them from their cages) did not significantly affect serum prolactin concentrations at any stages of the estrous cycle tested.

We next considered the effect of ether vapor plus serial blood sampling stress on serum prolactin levels (Fig. 3). Serum prolactin concentrations again were not in-

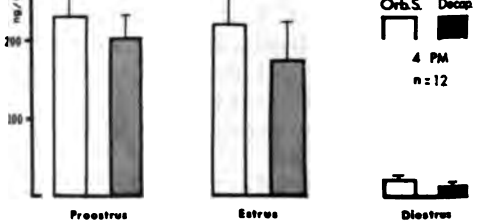
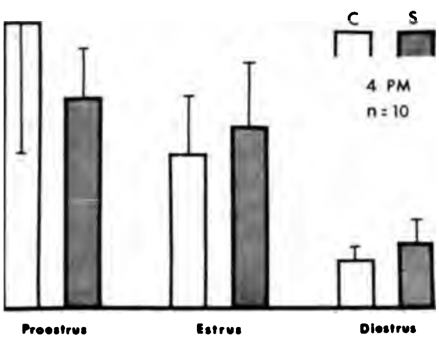


FIG. 1. Comparison of oribital sinus puncture and decapitation procedures of collecting blood for measurement of nonstressed levels of prolactin. Mean prolactin values and SEM are shown from blood collected at 4 PM at proestrous, estrous, and diestrous stages of the ovarian cycle.



2. Effect of serial ether vapor on rat serum
n at 4 PM on proestrous, estrus, and diestrus.
samples were taken by orbital sinus puncture
ight ether anesthesia. Rats were exposed to
t 1, 8, 15, and 25 min after cage disturbance.
perimental sample (S) was taken after the 25-
sthesia. Control samples (C) were taken from
rats not exposed to ether. Serum prolactin levels
are plotted as group means with indicated

d by this treatment in any of the
s tested. The high levels of prolactin
ntered on the afternoon of proestrus
strus were reduced by this treatment.
e other hand, the low level of prolactin
afternoon of diestrus Day 2 was not
ed by this treatment, and although
was a reduction in the mean prolactin
in the group stressed on the morning
us, these differences were not signifi-

effect of a single 2-hr restraint stress
ient on serum prolactin is shown in
. The high level of prolactin in the
rous group was again reduced by the
treatment. On the other hand, serum
tin levels were not affected by re-
stress in the estrous group, and the
ntrol group levels of prolactin in both
strate and diestrous group was sharply
sed following the 2-hr restraint stress
ient.

re 5 indicates the effect of 20 days of
c restraint stress on serum prolactin.
tin levels in blood samples before re-
was initiated on Days 1, 9, and 19
nated as control samples), increased
y during the experiment, rising from
242 ng/ml. Prolactin was increased in
ood sample taken at the end of the
ay of the restraint treatment. On the
hand, prolactin levels were less than
50 ng/ml at the end of the restraint period on Days 5, 10, 15, and 20. These end of stress prolactin levels are markedly lower than the pretreatment prolactin concentration measured on Days 9 and 19. Rats in this group were on the first day of diestrus when the treatment was started. This stress regime was sufficient to block regular ovarian cyclicity in all the experimental animals.

The effect of restraint stress on serum prolactin on the afternoon of the first day of pregnancy is depicted in Fig. 6. The 2-hr stress was administered to separate groups of rats from 12–2 PM or 3–5 PM. Serial blood samples were taken from each stress group and similar control rats before and after the stress treatment and at 1½ and 3 hr after the end of the stress regime. In the group receiving the stress treatment from 12–2 PM, prolactin levels were lower than control group concentrations only at the 3:30 PM sampling interval, 1½ hr after the end of the treatment. Prolactin levels in the 3–5 PM stressed group were less than control group concentrations at the end of the stress period, but were similar to control groups hormone levels at both 1½ and 3 hr after the end of the treatment.

The response of lactating rats to serial bleeding and restraint stress is depicted in Fig. 7. The pretreatment prolactin levels for both experiments were elevated. Both stress treatment regimes resulted in a prompt reduction in serum hormone levels. In the serial bleeding stress group, prolactin levels were only 1/3 as high by the 8-min sampling interval and continued to decrease until the end of the treatment. Prolactin levels in the group receiving the restraint treatment were reduced from 342 to only 68 ng/ml.

Discussion. These experiments indicate that the effect of stress on serum prolactin concentrations in the female rat varies markedly with the physiological state of the animal and the intensity of the stressor employed. Instead of uniformly stimulating prolactin secretion, in some instances stress regimes were found to have no effect on prolactin levels, and in other experimental situations stress was associated with a sharp reduction in serum prolactin. If the stress used was of relatively low intensity, such as periodic exposure to ether vapor alone at all stages of the estrous cycle we tested or ether

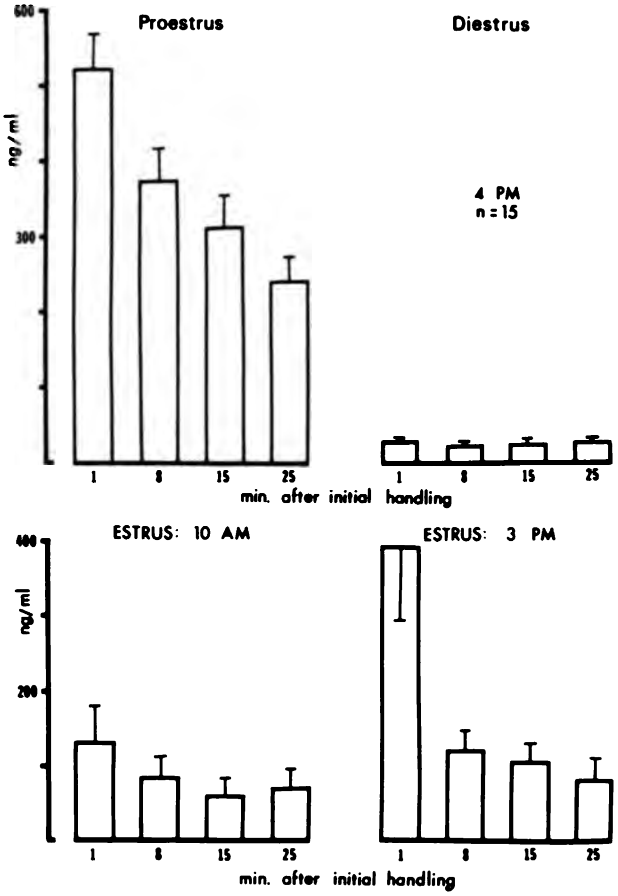


FIG. 3. Effect of ether vapor plus serial bleeding stress on rat serum prolactin at 4 PM on proestrus and diestrus and at 10 AM and 3 PM on estrus. Rats were ether anesthetized and serially bled by orbital sinus puncture at 1, 8, 15, and 25 min after cage disturbance. Serum prolactin levels (ng/ml) are plotted as group means with indicated SEM.

vapor plus serial bleeding stress in diestrous rats, serum prolactin concentrations were not affected. The effect of high-intensity stress was determined by the physiological state of the rat. Rats with high pretreatment serum prolactin concentrations, on the afternoon of proestrus, the afternoon of estrus, Day 1 of pregnancy, and during lactation, uniformly showed reduced prolactin levels after serial bleeding and all these groups except those on the afternoon of estrus had reduced serum prolactin following restraint stress treatment. These observations are in agreement with recent studies on the effects of ether stress on serum prolactin levels at proestrus (13) and the effects of serial bleeding on serum prolactin in lactating rats (15). On the other hand, rats with low pretreatment levels of prolactin, such as the diestrous and castrate groups, had increased serum prolactin levels following restraint stress. Thus, the initial level of prolactin in the serum appears to be important in determining the amount of prolactin released by a stress (13). Our Long-Evans rats show a diurnal peak of prolactin secretion in the afternoon of estrus similar to what we find on the afternoon of Day 1 of pregnancy (Fig. 6) and what has been previously reported on the first day of pseudopregnancy (16). The only group of rats that had lower serum prolactin levels on the afternoon of estrus than similar proestrus groups was that subjected to 2 hr of restraint stress (Fig. 4). It is of interest that this group which had initial serum prolactin levels inermediate between those of proestrous roups or other afternoon of estrous groups nd diestrous prolactin levels did not have ither increased or decreased serum prolacin after the restraint stress treatment.

These data do not indicate whether the hanges in serum prolactin related to stress re caused by modification of the rate of ituitary secretion, a change in metabolism r clearance of circulating prolactin, or a combination of both effects. A recent study considering the effects of ether stress during the proestrus surge indicated that the fall in serum prolactin during acute stress was similar to the decline after hypophysectomy (13). Another report suggests that the changes in serum LH following acute stress in the male rat are not due to alterations in metabolism or clearance of LH from the blood or to alterations in pituitary responsiveness to hypothalamic gonadotropin-releasing hormone (17). Current understanding of hypothalamic regulation of prolactin secretion implies that several hypothalamic factors may be involved. It has been suggested that prolactin secretion is regulated by both hypothalamic inhibitory factors (PIF) and stimulatory factors (PRF) (18, 19). Prolactin secretion has been reported to be influenced by alterations in hypothalamic content of serotonin (20), dopamine (21, 22), norepinephrine (23), and acetylcholine (24) affecting PIF and PRF secretion, or possibly to the hypothalamic secretion of dopamine and norepinephrine acting directly on pituitary prolactin secretion (25). Recent reports have shown that reserpine can block ether stress reduction of prolactin on the afternoon of proestrus (13) and that acute stressors can reduce arcuate nucleus content of both dopamine and norepinephrine (26).

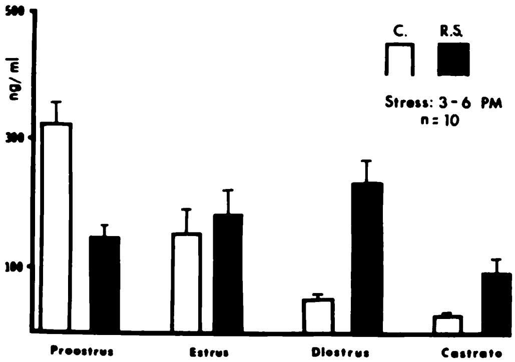


FIG. 4. Effect of 2-hr restraint stress on rat serum rolactin at proestrus, estrus, and diestrus, and in castrates. Control samples (C) were taken before restraint tress and the stressed samples (RS) at the end of the 2-r stress regime. Blood samples were taken by orbital inus puncture under light ether anesthesia. Serum proactin levels (ng/ml) are plotted as group means with ndicated SEM.

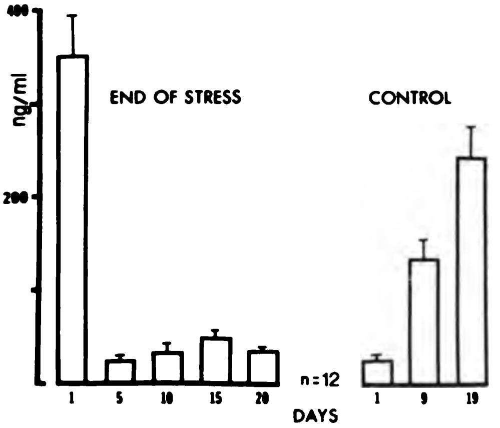


FIG. 5. Effect of chronic restraint stress (2 hr/day r 20 days) on serum prolactin. Prestress control sames were taken on Days 1, 9, and 19 of treatment. End stress samples were taken on Days 1, 5, 10, 15, and) of the stress regime. Blood samples were taken by bital sinus puncture under light ether anesthesia. rum prolactin levels (ng/ml) are plotted as group eans with indicated SEM.

It is clear from these experiments that serum concentrations of radioimmunoassayable prolactin can show prompt alterations with the initiation of stress treatments. If the alterations in serum prolactin are due to changes in hypothalamic PIF and PRF secretion, stress apparently affects their release differently in diverse physiological situations. When serum prolactin levels are high as during lactation or on the afternoon of proestrus, the effect of stress may involve either a decrease in PRF or an increase in PIF. On the other hand, the increased prolactin release seen after restraint stress in diestrous or castrate rats may be due to either an increase in PRF or a decrease in PIF. The effects of the different stressors on hypothalamic biogenic amines remain to be determined. In addition the sensitivity of the mechanisms controlling serum prolactin levels during stress was different in different physiological states. Pretreatment serum

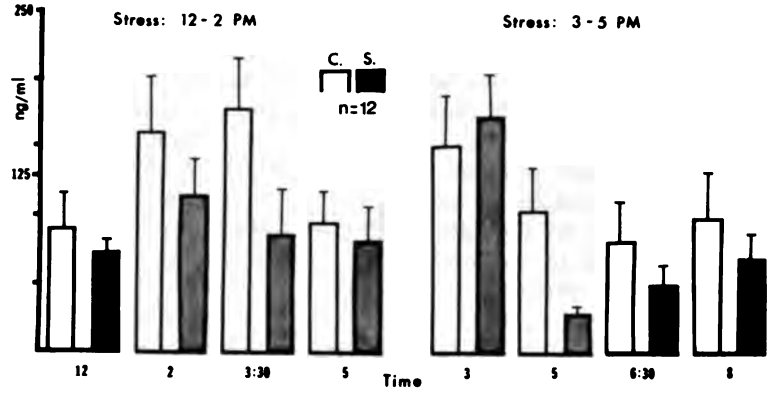


FIG. 6. Effect of restraint stress on serum prolactin on Day 1 of pregnancy. Rats received 2 hr of restraint stress from 12–2 or from 3–5 PM. Serial blood samples were taken from each rat before the stress, at the end of the stress, and 1 1/2 and 3 hr after the end of the stress. Blood samples were taken by orbital sinus puncture under light ether anesthesia. Serum prolactin levels (ng/ml) are plotted as group means with indicated SEM.

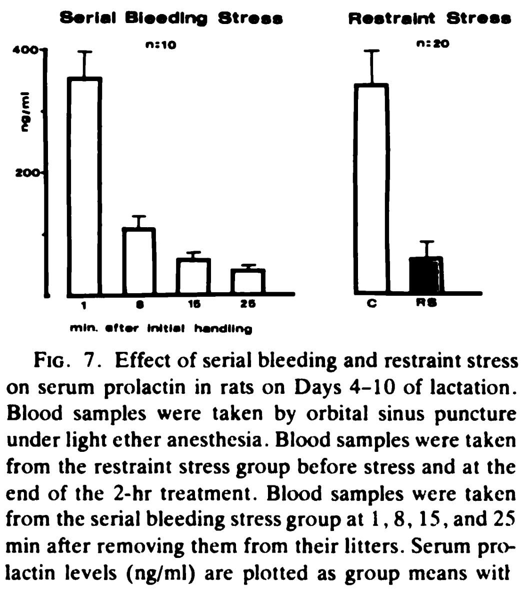


FIG. 7. Effect of serial bleeding and restraint stress on serum prolactin in rats on Days 4–10 of lactation. Blood samples were taken by orbital sinus puncture under light ether anesthesia. Blood samples were taken from the restraint stress group before stress and at the end of the 2-hr treatment. Blood samples were taken from the serial bleeding stress group at 1, 8, 15, and 25 min after removing them from their litters. Serum prolactin levels (ng/ml) are plotted as group means witl indicated SEM.

prolactin was elevated in the proestrous, estrous, pregnant, and lactating rats. The reduction of serum prolactin by stress treatments was greater in estrous rats subjected to serial bleeding and in the stressed lactating rats than after stress in the proestrous or pregnant groups and the estrous group subjected to the high intensity restraint stress. Although the latter had higher pretreatment prolactin levels than the diestrous group, their serum prolactin levels were lower than the proestrous group and they did not show any change in serum prolactin concentrations following the stress.

The effect of stress on serum prolactin on the first day of pregnancy also depended on the initial hormone levels. Serum prolactin levels of the control groups show a diurnal peak of serum prolactin at about 3 PM. The group of rats that received the stress treatment before the peak of serum prolactin (12–2 PM) did not show this diurnal peak and the group which received the stress during the diurnal peak (3–5 PM) had a prompt reduction of serum prolactin which was sustained throughout the sampling period.

The increase in pretreatment prolactin levels in the group of rats subjected to 20 days of restraint stress suggests that these rats were experiencing chronically increased prolactin levels through much of their treatment regime. On the other hand, the effect of the restraint stress on prolactin levels during the stress was markedly different during different periods of this treatment. On the first day of treatment, the elevated prolactin levels were typical of the data reported from the previous diestrous group exposed to this stressor. However, this high end of restraint stress prolactin level was not sustained during the remainder of the treatment period. The effect of restraint stress on this group of rats at Days 5, 10, 15, and 20 of treatment was to reduce an initially high prolactin level to the low end of treatment concentration, similar to the effect of this stressor on high pretreatment prolactin in other physiological states.

Although our data on the effect of simple exposure to ether vapor stress without serial bleeding are not in agreement with a recent report of decreased serum prolactin in proestrous rats following administration of this stress (13), our results from more intense stressors, serial bleeding with ether anesthesia and restraint stress, in groups of proestrous rats confirm this report. Although the sensitivity to stress may vary in different strains of rats and different investigators may alter the severity of a particular stress regime, these data reaffirm the importance of understanding the effects of stress on hormone control mechanisms, emphasize the importance of avoiding irregular or undue stress in animals used for studies of prolactin release, and point to the importance of maintaining uniform blood sampling times in experiments where hormone levels may be affected by stress.

Summary. Changes in serum prolactin in response to ether vapor, ether vapor plus serial bleeding, acute restraint and chronic restraint stresses were measured in cycling, castrate, pregnant, and lactating female Long-Evans rats. Serum prolactin was measured by radioimmunoassays in serial blood samples taken before and after administration of each stressor. Pretreatment prolactin levels were elevated in rats in the afternoon of proestrus and estrus, on Day 1 of pregnancy, and during lactation. Low initial prolactin concentrations were observed during diestrus, on the morning of estrus, and in castrate females. Low intensity stress, such as ether vapor alone, did not affect serum prolactin concentrations in any group tested. The effect of higher intensity stress was related to the pretreatment concentration of serum prolactin. If the initial prolactin levels were high, such as on the afternoon of proestrus and estrus, on Day 1 of pregnancy, and during lactation, high intensity stress was associated with a reduction in serum prolactin in all cases except for the group of rats on the afternoon of estrus which received the restraint stress treatment. On the other hand, if the pretreatment prolactin levels were low, such as in diestrous and castrate rats, high intensity stress was found to increase serum prolactin concentrations. Rats subjected to chronic restraint stess had increased pretreatment serum prolactin during the treatment regime. Although their initial prolactin concentrations were increased, these rats had lowered levels of prolactin at the end of their restraint periods. These data reaffirm the importance of understanding the effects of stress on hormone control mechanisms, and emphasize the importance of avoiding irregular or undue stress in animals used for studies of prolactin secretion.

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Adverse Reversible Effects of Chronic Ethanol Intake on Carbohydrate Metabolism (39416)

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(Introduced by W. C. Buchbinder)

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Several studies have shown that ethanol per se does not alter insulin secretion (1, 2). However, the data on the effects of ethanol on glucose-mediated insulin release and glucose tolerance are conflicting. For example, ethanol has been reported to delay or inhibit glucose-mediated insulin response as well as to induce glucose intolerance in humans and experimental animals (3-5). In contrast, other studies have shown an improvement in glucose tolerance and potentiation of glucose-mediated insulin release after ethanol pretreatment (6, 7). Metz *et al.* (6) have speculated about the use of ethanol as a therapeutic agent for the treatment of diabetes. The present study was designed to determine changes in carbohydrate metabolism after daily ingestion of ethanol for a period of 15, 30, and 60 days.

Materials and methods. Male Albino mice, weighing 20-25 g, were housed in conditions of controlled temperature, humidity, light cycle, and were fed Purina chow and tap water *ad libitum*. One week later, the mice were weighed, divided into three groups, with each group to receive one of the following solutions as the only drinking source: Group A, 10% v/v ethanol; Group B, 30% v/v ethanol; and controls, Group C, tap water for 15, 30, or 60 days. In order to check ethanol ingestion, blood ethanol levels were determined (8) in the fed state of randomly selected animals at different time intervals. Mean ± SE blood ethanol levels (mg/100 ml) were 32 ± 1.4 (n = 30) and 36 ± 1.8 (n = 30), respectively, in animals in Groups A and B. These values were not statistically different.

Oral Glucose Tolerance Test (GTT) was performed at the end of 15, 30, and 60 days of ethanol treatment. Mice were fasted for 18 hr except for water. In Groups A and B, ethanol was replaced by water to avoid the presence of ethanol in blood during the performance of GTT. A glucose load of 0.75 g/kg body weight dissolved in 0.2 ml of water per 10 g body weight was given by intragastric intubation. Some animals died during intubation resulting in different number of animals in each group. Blood samples were obtained from the orbital sinus before (0 time), and 30, 60, and 120 min after glucose administration. Whole blood was used for glucose measurements. For plasma insulin measurements, the microtubes were kept at 4° for 1 hr and then centrifuged for 3 min in a Beckman Spinco Microfuge. Plasma was separated and stored in plastic tubes at −20° for subsequent insulin assay. Blood glucose was determined on a Technicon AutoAnalyzer by the Hoffman ferricyanide method (9). Plasma immunoreactive insulin was determined by the double-antibody method of Morgan and Lazarow as modified by Blanks and Gerritsen (10). Plasma samples which showed hemolysis were discarded. Bovine insulin was used as a standard. Glucose increment ΔG, expressed as mg/100 ml/hr above its fasting value, and insulin increment ΔI, expressed as μU/ml/hr above its fasting value, were calculated. The insulinogenic index was determined by dividing ΔI by ΔG.

Three days after the performance of the GTT, peripheral insulin sensitivity was assessed in the same animals by determining the fall in blood sugar following the ip injection of insulin (0.2 U/kg) after an overnight fast. Blood samples for glucose estimation were obtained from the orbital sinus just before (0 time) and 30, 60, and 120 min after insulin injection.

To study whether the influence of chronic ethanol ingestion on carbohydrate metabolism was transient or permanent, 15 mice were given 10% ethanol as the only drink-

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ing solution for 30 days and subsequently tap water for another 30 days. An equal number of control animals received tap water. At the end of 60 days, GTT and insulin sensitivity tests were done on both groups of animals, as described above. The data was analyzed statistically by Student's *t* test.

Results. Figure 1 shows the influence of daily ethanol ingestion for 60 days on body weights of mice. There was not statistical difference in body weights of control and 10% ethanol-fed animals as determined at weekly intervals during the entire study period. Mice receiving 30% ethanol lost weight for the first 15 days and then gained weight, but to a significantly lesser degree ($P < 0.001$ at all points) than either the control or the 10% ethanol-treated animals.

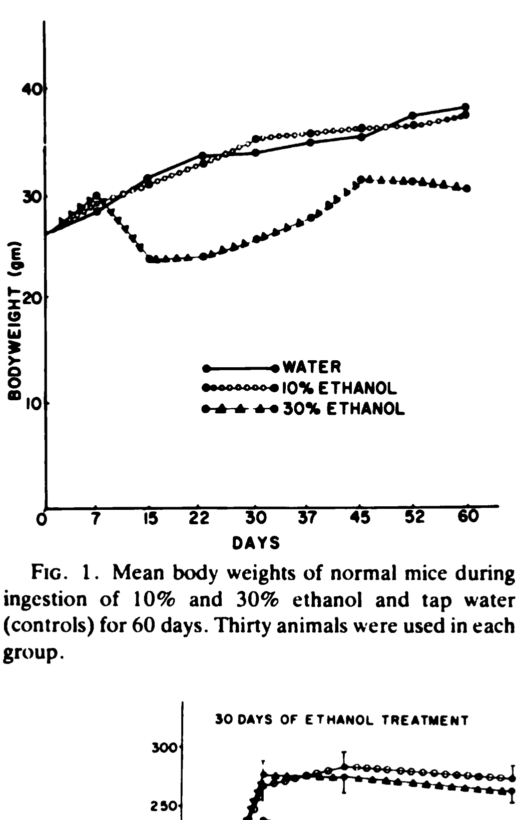

FIG. 1. Mean body weights of normal mice during ingestion of 10% and 30% ethanol and tap water (controls) for 60 days. Thirty animals were used in each group.

Effects of ethanol on oral glucose tolerance test. Fifteen days of daily ingestion of either 10 or 30% ethanol produced no discernible change in GTT as compared to control values (data not shown). Figure 2 shows the mean blood glucose response to oral GTT in mice fed 10% or 30% ethanol for 30 days. Fasting blood glucose values in ethanol-treated animals were not significantly different from those of the control animals. The respective values were 95 ± 3 (n = 24), 96 ± 4 (n = 26), and 86 ± 3 (n = 23) in control, 10% ethanol-, and 30% ethanol-treated animals. However, both groups of mice receiving ethanol showed abnormal glucose tolerance. Blood sugar values at 30, 60, and 120 min in the 10 and 30% mice were significantly different ($P < 0.01$) from the corresponding control values. Figure 2 also shows the effects of ethanol treatment for 60 days on GTT. The fasting blood sugar levels were not significantly different but the glucose tolerance tests were definitely abnormal in both ethanol-treated groups.

Effects of ethanol on plasma insulin. Figure 3 shows the mean plasma insulin response to oral glucose administration in control and ethanol-treated animals (n = 15 for each group of animals). Daily ingestion of 10 or 30% for 30 days did not exert any

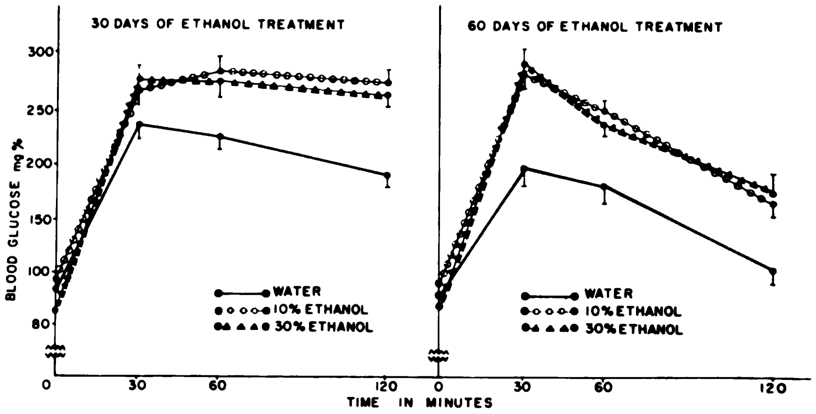

FIG. 2. Blood glucose values during oral glucose tolerance test in ethanol-fed and control mice. Means ± SE values are shown, and the number of animals in each group is as given in the text.

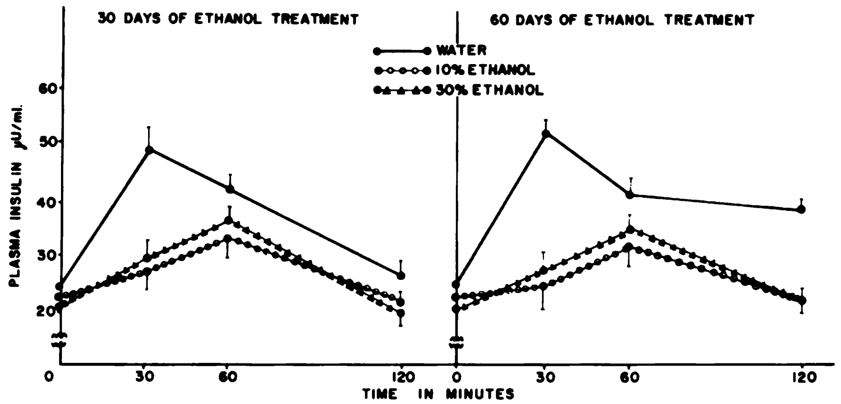

FIG. 3. Plasma insulin values during oral glucose tolerance test in ethanol-fed and control mice. Means ± SE values are shown and the number of animals in each group is as given in the text. Bovine insulin was used as standard for the plasma insulin determinations.

discernible effect on fasting plasma insulin levels. A significant decrease ($P < 0.01$ at all points) in plasma insulin response was observed at 30, 60, and 120 min in both ethanol-treated groups as compared to the respective control values. Peak insulin response to glucose was observed at 60 min in ethanol-fed animals and at 30 min in control animals. Figure 3 also shows that 60 days of 10 or 30% ethanol ingestion production essentially similar effects on plasma insulin levels. To see if the observed differences in insulin and glucose responses were interrelated, plasma insulin and blood glucose increments above the respective fasting values and the insulinogenic index were calculated (Table I). The insulinogenic index was significantly smaller ($P < 0.01$) in both groups of animals that received ethanol for 30 days than in the control animals (Table I). Similar changes in insulinogenic index were observed in animals that received ethanol for 60 days.

Effects of ethanol on insulin sensitivity test. Figure 4 shows the mean blood glucose response to insulin injection in control and ethanol-treated animals for 30 and 60 days. Both groups of animals that received ethanol were significantly ($P < 0.001$) less sensitive to insulin than the corresponding control animals. The magnitude of decreases in insulin sensitivity in the 10% ethanol-treated animals was not statistically different from that of the 30% ethanol-treated ones.

Effects of ethanol withdrawal on blood glucose and insulin levels. Figure 5 shows the mean blood glucose and plasma insulin responses to oral GTT in mice that were given 10% ethanol as the only drinking solution for 30 days and subsequently tap water for 30 days. In contrast to adverse effects of ethanol on GTT described above (Figs. 2 and 3), withdrawal of ethanol resulted in slight but significantly improved GTT as compared to that of control animals. Blood glucose increments were 124 ± 13 and 87 ± 3, $P < 0.01$ for the control and ethanol groups, respectively. There was no statistical difference in plasma insulin response between the two groups and the insulin increments were 24 ± 1 and 22 ± 1 μU/ml, respectively. Insulin sensitivity responses were also not statistically different as maximum decrease in blood glucose values were 29.1 ± 0.9 and 26.9 ± 1.1% of the respective baseline values in the ethanol-treated and control animals.

Discussion. Acute ethanol ingestion has been shown both to impair (3–5) and improve (6, 7) glucose tolerance in humans and experimental animals. The present study shows that chronic ethanol intake resulted in significant changes in glucose metabolism which include glucose intolerance, decreased and delayed glucose-mediated insulin response, as well as decreased peripheral insulin sensitivity. These changes were observed in animals that ingested ethanol as the only drinking solution for 30 or 60 days,

TABLE I. EFFECT OF DAILY ETHANOL INGESTION FOR 30 DAYS ON INSULINOGENIC INDEX IN NORMAL MICE.

Treatment	Blood glucose increment (mg/100 ml/hr)	Plasma insulin increment (μU/ml/hr)	Insulinogenic index $\frac{\Delta\text{Insulin}}{\Delta\text{Glucose}}$
Control	158 ± 13	22 ± 2	0.15 ± 0.02
10% Ethanol (15)[a]	246 ± 15[b]	7 ± 2[b]	0.03 ± 0.01[b]
30% Ethanol (15)	271 ± 11[b]	12 ± 3[b]	0.04 ± 0.01[b]

[a] Number of animals given in parentheses.

[b] $P < 0.01$ vs control. Results (Mean ± SE) represent increments of blood glucose and plasma insulin above the respective fasting values. Glucose tolerance test was done as described in the text.

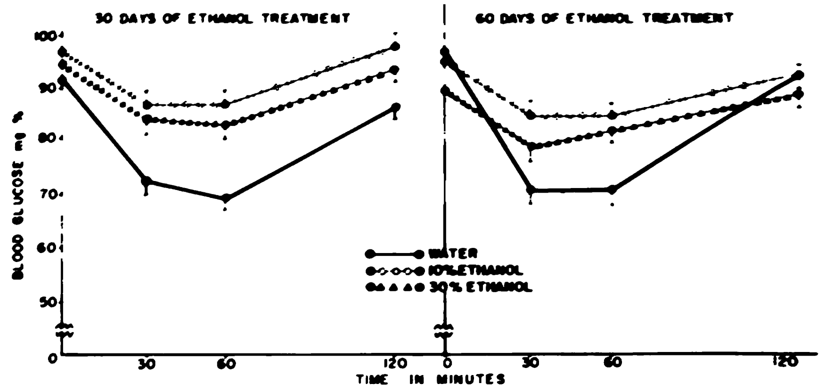

FIG. 4. Blood glucose values during insulin sensitivity test in ethanol-fed and control mice. Bovine insulin (0.2 U/kg) was given by ip injection. Means ± SE values are shown and number of animals in each group is as for Fig. 2 and given in the text.

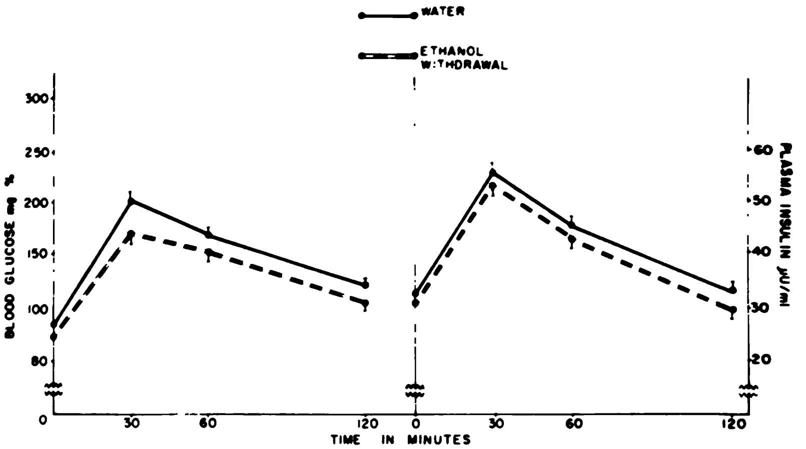

FIG. 5. Blood glucose and plasma insulin values during oral glucose tolerance test in 15 mice given 10% ethanol as the only drinking solution for 30 days and subsequently tap water for next 30 days. Age-matched 15 control mice received tap water for 60 days. Means ± SE values are shown. Bovine insulin was used as standard for the plasma insulin determinations.

but not when ethanol was given for 15 days. These experiments also indicate that the above-mentioned metabolic derangements were not permanent as withdrawal of ethanol improved or reversed various parameters to normal levels.

The data suggest that some of the factors (i.e., a decrease in glucose-mediated insulin

nse as well as in peripheral insulin sen-
y) may be responsible for ethanol-in-
d glucose intolerance. However, the
lying mechanisms by which ethanol in-
ces insulin secretion or peripheral insu-
ensitivity are unclear. First, ethanol
t exert direct effect on β-cells. *In vitro*
iments performed with pancreas of
rabbits, and golden hamsters have
n ethanol inhibition of glucose-me-
d insulin release (11, 12). Malaisse *et*
3) reported that ethanol interferes with
unctions of the microtubular system of
ls. Secondly, during a single transhe-
circulation, the liver normally retains
oximately 50% of the insulin pre-
d. An enhancement of insulin degrada-
y the liver because of ethanol might be
nsible for the diminished plasma insu-
sponse. However, except for the possi-
that the amount of insulin reaching the
itself affects the insulin uptake by liver
other factors which might influence
etention of insulin by the liver are not
established. Ethanol-induced hepatic
ge might be another reason for glucose
erance as derangement in carbohydrate
bolism occurs in chronic liver disease.
morphology was not studied in the
nt investigations. Another mechanism
t be an increase in blood levels of cate-
mines that can result from ethanol
tion. Catecholamines are known to in-
insulin secretion and action. Finally,
ol might alter insulin secretion via one
ore gut hormones that influence the
of Langerhans (15).

e significant decrease in peripheral in-
sensitivity in ethanol-treated animals is
rdant with the observations of Loch-
t *al.* (16) in dogs and Kreisberg (17) in
that ethanol has an inhibitory effect on
heral glucose utilization. Ethanol influ-
might be indirect via elevated free fatty
, adrenal corticosteroids and catechol-
es. Blood levels of these substances are
n to increase after ethanol ingestion
20). Whether adverse effects of ethanol
lucose metabolism are the results of
ol per se or one or both of its break-
products (acetaldehyde and acetate)
ot be ascertained from the present
Nonetheless, the observation that peripheral insulin sensitivity and diminished glucose-mediated insulin response improved or were restored to normal levels after ethanol withdrawal, suggests that the underlying mechanisms are reversible.

The findings of the present study may have implications concerning the use of ethanol in diabetic subjects. Some subjects have reported an improvement in glucose tolerance and potentiation of glucose-mediated insulin response after ethanol pretreatment (6, 7). This led to speculation about the use of ethanol as a therapeutic agent in diabetic humans. The present study showed adverse influence of chronic ethanol intake on glucose metabolism, albeit in normal mice. Daily ingestion of 10% and 30% ethanol induced essentially similar effects on glucose metabolism although the animals fed 30% ethanol lost weight as compared to those ingesting 10% ethanol.

Summary. The influence of chronic ethanol intake on carbohydrate metabolism was studied in mice. The results showed that daily ingestion of 10% or 30% ethanol for 30 or 60 days induced glucose intolerance, decreased and delayed glucose-mediated insulin response, as well as decreased peripheral insulin sensitivity. However, these metabolic derangements were not permanent as withdrawal of ethanol improved or restored these parameters to normal.

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ffect of Zinc Ions on Synthesis of Herpes Simplex Virus Type 2-Induced Polypeptides[1] (39417)

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ions at a concentration of 0.2 and M inhibit herpes simplex virus type 1 1) DNA synthesis by inhibiting virus polymerase (1). However, at a zinc centration of 0.1 m*M*, although virus synthesis was affected very little, only the virus was produced compared to treated controls (2). This indicates low concentration (0.1 m*M* or less), ns may affect other biological proc- nvolved in virus replication and may y inhibit virus progeny formation. ns have been shown to inhibit post- tional processing of the precursor ptides of rhinovirus and other picor- structural proteins (3, 4). Thus, it interest to examine the effect of zinc herpesvirus-induced polypeptides in d cells. Herpes simplex virus type 2 2) was selected for this study since ll protein synthesis is depressed much in HSV-2-infected than in HSV-1- d cells (5). The present study demon- that zinc ions at a concentration of M inhibit the synthesis of virus-in- polypeptides with little effect on virus synthesis.

rials and methods. Cells and viruses. ells were propagated as monolayer s at 37° in medium 199 supple- with 10% fetal calf serum (FCS). strain 333 was grown in Vero cells d throughout this investigation.

synthesis. Vero cells were infected SV-2 strain 333 at a multiplicity of n (m.o.i.) of 3-5. One hour postin- , cells were incubated with medium th 5% FCS in the absence or pres- 0.05, 0.1, or 0.2 m*M* $ZnSO_4$. At 4 infection, [^{3}H]thymidine was added to the medium to a final concentration of 10 μCi/ml. The infected cultures were incubated for 10 hr at 37°, harvested by scraping the cells into the medium, and centrifuged at 750 *g* for 10 min. The cell pellet was then suspended in 0.1 × SSC (0.15 *M* NaCl, 0.015 *M* Na-citrate) containing 1% (w/v) sarkosyl and 1 mg/ml of pronase, and incubated at 37° for 12–14 hr. The digested sample was then analyzed by CsCl density gradient centrifugation at 32,000 rpm for 60 hr in a 40.3 rotor. Drops were collected from the bottom and TCA-precipitable radioactivity was determined. Densities were determined by measuring refractive index.

Protein synthesis. Labeling of virus-induced polypeptides was accomplished using the procedure described above, except that at 3 hr postinfection, medium was replaced with the labeling medium containing [^{35}S]methionine in the absence or presence of $ZnSO_4$. Labeling medium consisted of medium 199 containing one-half the normal concentration of essential amino acids, 5% FCS, and 5 μCi/ml of [^{35}S]methionine (sp act 360 Ci/mmole, Amersham Searles, Inc.). Infected cells were harvested 18 hr after infection and lysed with a buffer containing 0.0625 *M* Tris (pH 6.8), 0.4% Triton X-100, 10 m*M* $MgCl_2$, 50 μg/ml each of DNase and RNase, and 300 μg of methyl phenyl sulfonyl fluoride. The mixture was incubated for 10 min at room temperature, after which sodium dodecyl sulfate (SDS) and mercaptoethanol were added to a final concentration of 2 and 5%, respectively. After heating at 100° for 2 min, 50 μl of each mixture was analyzed by 5–15% gradient polyacrylamide slab gel electrophoresis in the presence of SDS, as described by Baum *et al.* (6). Following electrophoresis, the gel was dried and an autoradiogram was prepared using sarkosyl and pronase and analyzed by CsCl gradient centrifugation.

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served.

Densities were determined to distinguish virus DNA from cellular DNA. Labeling of virus-induced polypeptides was accomplished using the procedure described above, except that at 3 hr postinfection, medium was replaced with the labeling medium containing [^{35}S]methionine in the absence or presence of $ZnSO_4$. The cultures were harvested at the end of the virus growth cycle (18 hr postinfection) and lysed. The proteins were solubilized with SDS and mercaptoethanol and analyzed by 5-15% gradient polyacrylamide slab gel electrophoresis. Following electrophoresis, the gel was dried and an autoradiogram was prepared.

Results. Before attempting to determine the effect of zinc ions on virus replication, we examined their effect on host cells. Zinc ions at a concentration of 0.1 m*M* and higher were found to be toxic, resulting in rounding up of cells and detachment from the glass wall under conditions which slowed cell growth. This occurred with confluent cells and with serum-free or essential amino acid-free media. Hence, all experiments were carried out on subconfluent (70-80% confluent) cells in medium containing at least 5% FCS and one-half the normal concentration of all essential amino acids. Even under these conditions, however, zinc ions were found to be toxic at a concentration of 0.3 m*M*.

The effect of zinc ions on formation of HSV-2 virus progeny was measured. Zinc ions at a concentration of 0.05 m*M* and higher inhibited virion synthesis (Table I), a finding similar to that of Gordon *et al.* (2) with HSV-1. Removal of zinc ions 4 hr postinfection restored the virus yield to almost equal that of the untreated control.

The effect of various concentrations of zinc ions on the synthesis of HSV-2 and cellular DNA was determined. The infected untreated and zinc-treated cells were labeled for 10 hr (4-14 hr postinfection) with [^{3}H]thymidine. Labeled DNA was then analyzed by centrifugation in CsCl density gradients (Fig. 1). The amount of radioactivity banding at a density of 1.725 g/ml (virus DNA) and 1.698 g/ml (cellular DNA) was determined and plotted against various concentrations of zinc sulfate (Fig. 2). Zinc ions to a concentration of 0.1 m*M* had a slight inhibitory effect on the synthesis of virus DNA, while at a concentration of 0.2 m*M*. the synthesis of virus DNA was markedly inhibited. In the presence of 0.1 m*M* zinc ions, although DNA synthesis was inhibited only slightly, marked inhibition of virus yield was observed.

To determine the effect of zinc ions on HSV-2-induced polypeptides, infected cells were labeled with [^{35}S]methionine for 15 hr (3-18 hr postinfection) at various zinc ion concentrations. Figure 3 shows analysis by SDS-polyacrylamide gel electrophoresis of labeled virus-induced polypeptides. Zinc ions at a concentration of 0.2 m*M* had no obvious effect on host cell protein synthesis (lanes a and b). At a concentration of 0.05 m*M*, the zinc ions slightly inhibited the synthesis of virus-induced polypeptides, particularly polypeptide P_1 with an approximate

TABLE I. EFFECT OF ZINC IONS ON REPLICATION OF HSV-2.

$ZnSO_4$ (m*M*)	Virus yield[a] (% of control)
0	100
0.05	10
0.1	5
0.2	<0.1
0.1 for 4 hr, then washed	100

[a] $ZnSO_4$ was added 1 hr postinfection. Eighteen hours later, infected cells were scraped into the medium, frozen and thawed three times, and the resulting virus was assayed in rabbit kidney cells. Number of plaques found in untreated control cultures was regarded as 100%.

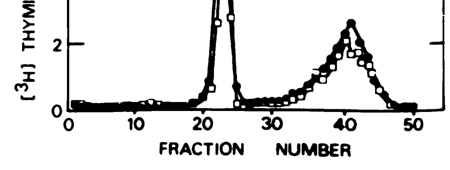

FIG. 1. Analysis of DNA from HSV-2-infected Vero cells in the presence of 0.1 m*M* $ZnSO_4$ (-●-●-) and in the absence of $ZnSO_4$ (-□-□-) by isopycnic centrifugation in CsCl density gradient.

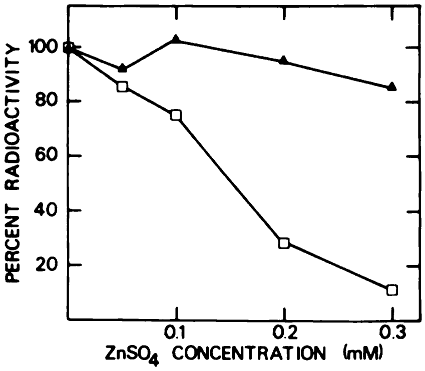

FIG. 2. Effect of zinc ions on the synthesis of virus d cellular DNA in HSV-infected cells. The infected lls were either untreated or treated with different ncentrations of $ZnSO_4$. The cultures were labeled th [^{3}H]thymidine and the DNA was analyzed by CsCl nsity gradient centrifugation. The amount of radiotivity banding at a mean density of 1.725 g/ml (virus NA) (-□-□-), and 1.698 g/ml (cellular DNA) (-▲-), was determined and plotted against various contrations of $ZnSO_4$ used. The DNA (virus or cellu) obtained from infected untreated cells (no $ZnSO_4$) s regarded as 100%, and virus and cellular DNA m the $ZnSO_4$-treated cells were compared to it.

molecular weight of 130,000 (lane d), when compared to untreated controls (lane c). The inhibition was more pronounced in the presence of 0.1 m*M* of $ZnSO_4$, when most of the polypeptides were synthesized in reduced amounts (lane e), particularly polypeptides P_1, P_2, P_3, and P_4 (P_2 = 88,000 mol wt; P_3 = 58,000 mol wt; P_4 = 38,000 mol wt). At a concentration of 0.2 m*M* $ZnSO_4$, very little virus-induced protein was synthesized (lane f). Unlike with picornaviruses, no accumulation of large virus polypeptides was noticed in zinc-treated infected cells. The inhibitory effect of zinc could be removed by washing the infected cells up to 4 hr postinfection, which resulted in the resumption in normal amounts of virus-induced polypeptide synthesis (lane g) and the production of normal amounts of virus progeny (Table I). Similar inhibition of virus-induced polypeptides was also observed in HSV-2 grown in human embryo lung cells (data not shown).

Discussion. The present study demonstrates that $ZnSO_4$ at a concentration of

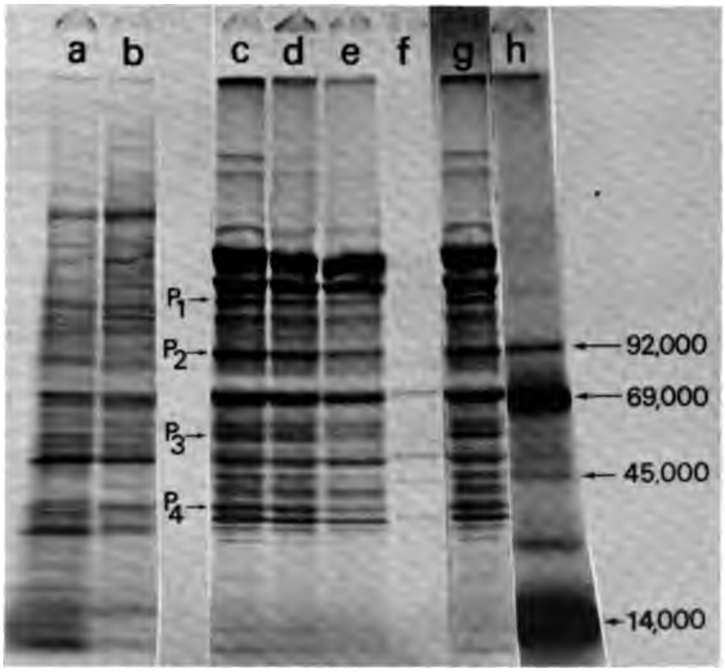

FIG. 3. Polyacrylamide gel electrophoresis analysis of the polypeptides synthesized in infected untreated and c-treated cells. Lane a, uninfected culture; b, uninfected culture treated with 0.2 m*M* $ZnSO_4$; c, infected culture) $ZnSO_4$); d-f, infected cultures treated with 0.05, 0.1, and 0.2 m*M* $ZnSO_4$; g, infected culture exposed to 0.1 *M* of $ZnSO_4$ for 4 hr, after which it was removed; h, standard proteins of known molecular weight indicated by ows.

0.05 and 0.1 m*M* prevents the synthesis of virus-induced polypeptides, especially polypeptides P_1, P_2, P_3, and P_4, and also prevents virus progeny formation. In contrast, 0.1 m*M* or less $ZnSO_4$ has little effect on virus DNA synthesis. In the presence of 0.2 m*M* $ZnSO_4$, synthesis of both virus DNA and protein is markedly inhibited. It has been shown that $ZnSO_4$ at a concentration of 0.2 m*M* prevents the synthesis of HSV DNA by inhibiting virus DNA polymerase activity. Zinc ions have no effect on the synthesis of virus DNA polymerase (1). Thus, at 0.2 m*M* $ZnSO_4$, in addition to an inhibitory effect on synthesis of specific virus-induced polypeptides, drastic reduction in virus DNA synthesis probably results in a marked inhibition of virus-specific transcription and, hence, of synthesis of total virus-induced polypeptides. The inhibitory effect of zinc ions could be reversed by removing zinc ions up to 4 hr postinfection, a finding contradictory to that of Gordon *et al.* (2), who reported the inhibitory effect to be irreversible with HSV-1 and BSC-1 cells. This contradiction could be due to the difference in cells and virus used. Since certain virus-induced polypeptides are virion structural proteins, it is conceivable that inhibition of synthesis of one or more of the polypeptides will also inhibit virus progeny formation. The mechanism by which zinc ions inhibit herpesvirus-induced polypeptides has not yet been determined.

Summary. Treatment of herpes simplex virus-infected cells with zinc sulfate ($ZnSO_4$) inhibited formation of virus progeny, but had little effect on virus DNA synthesis. Analysis of polypeptides synthesized in infected cells in the presence of $ZnSO_4$ revealed a markedly reduced synthesis of virus-induced polypeptides.

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n the Intracellular Localization of the 3β-Hydroxysteroid Dehydrogenase/Isomerase in the Rat Adrenal Cortex[1] (39418)

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troversy exists with regard to the ex-
racellular localization of the 3β-hy-
steroid dehydrogenase/isomerase
)[4] in steroid hormone-producing cells.
early report, Beyer and Samuels (1)
that the enzyme was distributed
all subcellular fractions derived from
enates of beef adrenal cortex but that
crosomal fraction had the highest spe-
ctivity. Since then, HSD has been
purified from sheep adrenal cortex
omes (2) and is now generally ac-
to be a constitutive microsomal
rane-bound enzyme. On the other
several reports have suggested that
s also a mitochondrial enzyme in the
l cortex (3), testis (4), ovary (5), and
ta (6). Investigators seeking to re-
his descrepancy have variously con-
that mitochondrial HSD results from
t microsomal contamination (7); or
n enzyme redistribution artifact that
during homogenization (8); or from
al mitochondrial localization (4). If
s located in the inner mitochondrial
ane, as suggested by Sulimovici *et al.*
roid oxidation might proceed utiliz-
D contained in the matrix space. In
ort we show that this reaction does
nd conclude that HSD is present in
er mitochondrial membrane in rat
l cortex mitochondria.

Materials and methods. Male Sprague-Dawley rats weighing 150–250 g were used in these experiments. The adrenals from 10 to 15 animals, sacrificed by decapitation, were removed and chilled in cold isotonic sucrose. The adherent fat was removed and the glands were homogenized in 0.25 *M* sucrose containing 1 m*M* EDTA and 30 m*M* Tris-HCl, pH 7.2. A mitochondrial fraction was isolated as previously described (9). The crude mitochondrial pellet was washed three times by resuspension and recentrifugation. Microsomes were isolated from the postmitochondrial supernatant fluid by centrifugation for 1 h at 105,000 *g*. The microsomal and washed mitochondrial pellets were resuspended in homogenizing medium to total volumes of 2 ml.

All incubations were performed at 37° in duplicate 1-ml portions of a medium that contained 50 m*M* sucrose, 20 m*M* KCl, 1 m*M* EDTA, 30 m*M* Tris-HCl, pH 7.4, and 0.2 to 0.5 mg of mitochondrial or microsomal protein. HSD activity was measured as described by Neville and Engel (10). For this assay the medium also contained 0.1 m*M* [^{3}H]pregnenolone (30,000 dpm). NAD, when present, was 0.5 m*M* and other additions were as indicated in the tables. Blank incubations contained no enzyme. The reaction was started by the addition of [^{3}H]pregnenolone and was stopped, after 5 min, by the addition of either 2 ml of heptane or 10 ml methylene chloride. [^{3}H]Progesterone formation was constant with time for 5 min and with tissue protein to 0.7 mg. The 21-hydroxylase activity was measured by the method of Koritz and Kumar (11). The medium also contained 0.1 m*M* [^{3}H]progesterone (30,000 dpm), 50 m*M* NADPH, 3.3 m*M* glucose-6-phosphate, and 2 IU glucose-6-phosphate dehydrogenase. The reaction was started by the addition of progesterone and was stopped,

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operstown, New York 13326.
abbreviation used is: HSD, 3β-hydroxysteroid
enase/isomerase.

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erved.

after 10 min, by the addition of 2 ml of heptane. The 11β-hydroxylase activity was measured by the conversion of deoxycorticosterone to corticosterone as previously described (9). For this assay the medium also contained 30 μM [^{3}H]deoxycorticosterone. Reducing equivalents were supplied by either 10 mM malate or isocitrate or an NADPH-regenerating system. The reaction was started by the addition of deoxycorticosterone and was stopped, after 5 min, by the addition of 10 ml of methylene chloride.

In the three enzyme assays, aliquots of ^{14}C-labeled steroid substrates and products were added prior to extraction to correct for losses of tritiated steroid during the extraction and the chromatographic separations. The incubates were extracted twice and the organic phases were separated and evaporated to dryness. Carrier, unlabeled steroids were added to enhance visualization by UV absorption. Thin-layer chromatography was performed on Silica gel G Plates using the following solvent systems: pregnenolone and progesterone, either chloroform–methanol (99:1) or chloroform–ether (9:1); progesterone and deoxycorticosterone, either chloroform–methanol (99:1) or ether–benzene (9:1); and deoxycorticosterone and corticosterone, ether–benzene (9:1). The substrates and products of the 21-hydroxylase reaction were also separated on a 1 × 39-cm column of Sephadex LH-20 (11 g) as described by Tan and Mulrow (12). After elution from either the thin-layer plates or the Sephadex LH-20 columns, the radioactive steroids were counted in a Packard Tri-Carb scintillation counter. Quenching was estimated by internal standardization.

Results and discussion. Mitochondrial and microsomal HSD activity. HSD was assayed in mitochondria and microsomes prepared from several different rat adrenal homogenates. The mean specific activities (± SEM) are summarized in Table IA. While HSD activity was present in both the mitochondrial and microsomal fractions the specific activity of HSD was higher in the microsomes only in the presence of added NAD. This resulted from the fact that the mitochondrial HSD was active in the absence of added NAD. Exogenous NAD about doubled the mean mitochondrial HSD activity. In contrast, the microsomal HSD activity was low in the absence of NAD and was increased at least 20-fold by exogenous NAD. As shown in Table IB, these mitochondrial preparations contained measurable amounts of 21-hydroxylase, a microsomal-marker enzyme in this tissue (7). In some preparations (not shown) 21-hydroxylase activity reached values as high as 3 nmol deoxycorticosterone formed per minute per milligram of mitochondrial fraction protein. However, the ratio of HSD activity (measured in the presence of NAD) to the 21-hydroxylase activity was five to seven times greater in the mitochondrial fraction

TABLE I. THE SPECIFIC ACTIVITIES OF 3β-HYDROXYSTEROID DEHYDROGENASE (HSD) AND 21-HYDROXYLASE IN RAT ADRENAL MITOCHONDRIAL AND MICROSOMAL FRACTIONS.

Fraction	HSD activity[a] No NAD	HSD activity[a] 0.5 mM NAD	21-Hydroxylase activity[a]	HSD (with NAD) / 21-Hydroxylase
A				
Mitochondria (12)	7.5 ± 1.0	14.4 ± 1.2	–	–
Microsomes (5)	1.1 ± 0.4	26.5 ± 4.9	–	–
B				
Mitochondria (2)	13.5	20.5	1.0[b]	21
Microsomes (2)	2.1	33	10.1	3.4

[a] The enzyme assays were performed as described under Materials and Methods. The activities are in nanomoles progesterone (HSD) and deoxycorticosterone (21-hydroxylase) formed per minute per milligram of protein. The numbers in parentheses represent the number of separate experiments. Some variation in enzyme specific activity was noted among different preparations but the relationships shown under A and B always held.

[b] 11β-Hydroxyprogesterone may be formed from progesterone by mitochondria. This steroid, which migrated with deoxycorticosterone in the thin-layer chromatographic system (ether:benzene, 9:1) used for the 21-hydroxylase assay in microsomes, was separated from deoxycorticosterone by thin-layer chromatography using chloroform:methanol (99:1) or by Sephadex LH-20 chromatography (as described in Methods).

ıred to the microsomal fraction. We ret this finding to mean that the HSD y of the mitochondrial fraction cannot e entirely to microsomal contamina- Similar HSD/21-hydroxylase activity were found by Moustafa and Koritz rat adrenal mitochondrial and micro- fractions, but were interpreted to a selective contamination of the mito- ria by microsomes high in HSD and ı 21-hydroxylase activity. To our edge such a heterogeneity of enzyme y has not been demonstrated in the ıl cortex endoplasmic reticulum.

:e about half of the mitochondrial activity appeared to result from an membrane enzyme with an active site the matrix space, an attempt was to influence the activity of this enzyme uction of the intramitochondrial pyri- ucleotides. We have previously shown hat a combination of inorganic phos- citric acid cycle substrates, and inhibi- ɔf mitochondrial electron transport l a reduction of the pyridine nucleo- n these mitochondrial preparations. II shows that this reagent combina- hibited endogenous NAD-dependent ıondrial HSD activity about 90%. ates plus inorganic phosphate alone ed about 75% and rotenone and cya- lone inhibited only about 10% (not). In the presence of exogenous mitochondrial HSD activity was de- d about 40% on addition of the inhib- ıbstrate combination. Microsomal vas essentially inactive in the absence genous NAD and the inhibitor-substrate combination did not inhibit microsomal HSD activity significantly in either the presence or absence of NAD.

The [^{3}H]progesterone formed during the mitochondrial and microsomal HSD reactions was characterized in two different thin-layer and a Sephadex LH-20 chromatographic system (see Materials and Methods). Recovery of added tracer [^{14}C]progesterone ranged from 88 to 113%. In other experiments (not shown), we found good correlation between pregnenolone disappearance and progesterone formation. Therefore, no new or different products were formed during the mitochondrial HSD reaction.

On the basis of these results it seems reasonable to conclude that the mitochondrial progesterone formation that was inhibited by rotenone, KCN, and the citric acid cycle substrates was dependent on intramitochondrial NAD. We assume that the exogenous NAD-dependent HSD activity of these mitochondrial fractions is due either to microsomal contamination and/or to damaged mitochondria whose permeability barriers to exogenous NAD have been impaired. Moustafa and Koritz (7) reported that about one-half of the mitochondrial HSD could be accounted for by microsomal contamination. In our experiments (Table I), microsomal contamination accounted for less than 20% of the mitochondrial HSD activity. A redistribution of HSD from microsomes to mitochondria, as suggested by Cowan *et al.* (8), seems unlikely because this would require the selective transfer of HSD and not the 21-hydroxylase. A transfer of HSD from

E II. THE EFFECT OF A COMBINATION OF KREBS CYCLE SUBSTRATES AND RESPIRATORY INHIBITORS ON HSD ACTIVITY IN MITOCHONDRIA AND MICROSOMES.

	HSD activity[a]			
	No NAD		0.5 m*M* NAD	
		+Inhibitors[b]		+Inhibitors[b]
ochondria (6)	5.8 ± 1.1	0.60 ± 0.20	12.0 ± 1.3	7.7 ± 1.2
rosomes (2)	0.40 ± 0.04	0.67 ± 0.11	16.1 ± 4.9	21.5 ± 7.1

ıochondrial and microsomal HSD activity was measured as described in Materials and Methods in either ence or absence of 0.5 m*M* NAD. HSD activity is given as nanomoles progesterone produced per minute igram of protein ± SEM. The numbers in parentheses represent the number of different experiments.

: inhibitor combination used was 10 m*M* isocitrate, 10 m*M* α-ketoglutarate, 5 μ*M* rotenone, 0.5 m*M* , and 1 m*M* phosphate. Analysis of the entire thin-layer chromatographic plate showed that no steroid an progesterone was formed during these incubations, i.e., the inhibitor-dependent decrease in progester- nation was not due to the appearance of 11β-hydroxyprogesterone.

microsomes to the inner mitochondrial membrane so that the active site faced the matrix space without disruption of the mitochondria is especially difficult to imagine.

The role of mitochondrial HSD in adrenal cortex steroidogenesis is purely speculative at this point. If the mitochondrial enzyme is regulated *in vivo* by the NADH/NAD ratio of the matrix space as suggested here, the product of cholesterol side chain cleavage that leaves the mitochondrion could be either pregnenolone or progesterone. Hochberg and Lieberman (14) recently reported that progesterone is a major product of cholesterol side chain cleavage in rat adrenal mitochondria oxidizing succinate. Since NAD was not added in their experiments they suggested that HSD was present in the mitochondria. Our results support that interpretation. Further studies designed to clarify the effect of the intramitochondrial pyridine nucleotide redox state on the product of cholesterol side chain cleavage will be of interest.

Summary. In this study we have compared some properties of the microsomal and mitochondrial 3β-hydroxysteroid dehydrogenase/isomerase (HSD) from the rat adrenal cortex. The following major differences were noted: (i) the microsomal enzyme required exogenous NAD while the mitochondrial enzyme was active in the absence of added NAD. (ii) The mitochondrial enzyme was inhibited by a combination of rotenone, KCN, and citric acid cycle substrates. These agents did not influence the activity of the microsomal enzyme. (iii) The ratio of the specific activities of HSD to the 21-hydroxylase (a microsomal marker enzyme) was greater in the mitochondria than in the microsomes. We conclude that HSD is present in the inner mitochondrial membrane and, therefore, that the enzyme has a dual mitochondrial and microsomal localization in the rat adrenal cortex.

The authors wish to thank Drs. P. J. Mulrow and S. Y. Tan for several helpful discussions during the course of this study.

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w Aldehyde Dehydrogenase Isozymes in Chemically Induced Liver Tumors in the Rat[1] (39419)

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have earlier reported (1, 2) that liver
induced in Sprague-Dawley rats by
ding of acetylaminofluorene (AAF)
hibit new isozymes of aldehyde dehy-
ase (Ald D). These new forms can be
uished from those of normal rat liver
eral criteria: (a) normal Ald D is
specific on polyacrylamide gels, while
Ald D can use either NAD or
; (b) normal Ald D isozymes have pI
in the range of 6.2-6.4, whereas
f liver tumors have a neutral pI (pH
2); (c) the new forms are all cytoplas-
hereas normal liver Ald D is distrib-
etween cytoplasm and particulate
s; and (d) the tumor isozymes are
table than the normal forms to heat,
remes, guanidine, and urea. The pur-
f the present communication is to
e the relationship of the new Ald D
o species, strain, tissue, and carcino-
gent. Liver tumors discussed in this
range from those sometimes called
plastic nodules" (3) to malignant ad-
cinoma; it was found that neither the
isozyme pattern nor the total enzyme
correlated with the degree of cellu-
erentiation.

rials and methods. Animals used
eanling male Sprague-Dawley and
rats. They were fed the diets de-
by Peraino *et al.* (4): (a) normal diet
casein); (b) normal diet containing
phenobarbital; or (c) normal diet
ing 0.02% AAF or DAB (dimethyl-
zobenzene). Three dietary schedules
mployed to induce liver tumors: (a)
r DAB diet for 18 days, then normal
ereafter; (b) AAF for 18 days, then
arbital diet thereafter; or (c) four
cycles, each consisting of 4 weeks on AAF diet, followed by 1 week on normal diet. This last regimen resembles that described by Reuber (5).

Liver tumors were also induced with ethionine in a manner similar to that described by Epstein *et al.* (3). Weanling Sprague-Dawley male rats were fed a diet containing 0.25% ethionine for 5 weeks, then a diet containing 0.5% ethionine for 3 weeks, and finally a diet containing 0.8% ethionine for 8 weeks, followed by a normal diet thereafter.

Ald D activity was assayed as described earlier (1); essentially, this assay measures the increase in A_{340} due to reduction of NAD or NADP to the reduced form, as benzaldehyde is oxidized. Polyacrylamide gel electrophoresis (PAGE) was done using the Canalco apparatus and reagents, and isoelectric focusing on polyacrylamide gel slabs was performed with the Multiphor apparatus (LKB Produkter, Bromma, Sweden), modified for greater flexibility as decribed elsewhere (6).

Results. In Sprague-Dawley (S.-D.) rats we were able to induce liver tumors by AAF or DAB by any of the dietary regimens of Peraino described above (Table I), and the tumors invariably resulted in the production of the new isozyme forms of Ald D. In Buffalo rats liver tumors could be produced by AAF only by the four-cycle regimen of Reuber (5); however, all liver tumors produced in a Buffalo rat by AAF invariably showed the new Ald D isozyme forms having the characteristics described in the introduction.

After ethionine feeding, considerably fewer tumors developed. These tumors, with a single exception, were lower in Ald D activity than normal liver, and showed none of the characteristics of the Ald D of AAF

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served.

or DAB tumors. The single exception was a late developing hepatocellular carcinoma which showed Ald D isozymes of high activity and characteristics similar to those in AAF- or DAB-induced tumors.

Table II indicates that the high Ald D activity observed in AAF- or DAB-induced liver tumors is not found in some other liver tumors tested, including several Morris hepatomas, or in a variety of tumors of other tissues.

Table III describes the Ald D activity of nontumorous rat liver. Particularly noteworthy is the fact that, unlike the more common situation with other isozymes in liver tumors, the Ald D activity of the tumor does not recapitulate the fetal liver pattern. Not only is the total Ald D activity of fetal liver much lower than in the AAF and DAB liver tumors, but the fetal enzyme has a specific requirement for NAD and has a pI like that of the normal liver isozymes.

Figure 1 compares the isoelectrically focused Ald D isozyme patterns of AAF, DAB, and ethionine tumors, and of normal liver, all with NAD as coenzyme. It is of interest to note that (a) the lower pI isozymes (pH 6.2–6.4) of normal liver are still expressed by the tumors induced by AAF and DAB; (b) the AAF and DAB tumor isozymes appear qualitatively similar (pI 6.9–7.2), although in this particular instance there is some quantitative difference; and (c) the ethionine tumor (a hepatocellu-

TABLE I. Aldehyde Dehydrogenase Activity of Rat Liver Tumors as Related to Rat Strain and Carcinogen Fed.[a]

Rat strain	Tissue	Dietary regimen	Ald D activity[b] Number	Ald D activity[b] Mean ± SD	Ald D activity[b] Range
S.-D.	Normal liver	Normal diet	15	65 ± 14	39-88
S.-D.	Liver tumor	AAF, then normal	6	215 ± 106	74-355
S.-D.	Liver tumor	AAF, then phenob.	10	239 ± 94	91-390
S.-D.	Liver tumor	AAF, 4 cycles	33	187 ± 348	14-2010
S.-D.	Liver tumor	DAB, then phenob.	3	374 ± 128	228-436
S.-D.	Liver tumor	Ethionine	28	17 ± 13	0-48
S.-D.	Liver tumor	Ethionine	1[c]	152	
Buffalo	Normal liver	Normal diet	3	68 ± 6	62±73
Buffalo	Liver tumor	AAF, 4 cycles	58	129 ± 168	0-658

[a] Abbreviations used: SD, standard deviation; S.-D., Sprague-Dawley; phenob, phenobarbital.

[b] Ald D activity units are defined as (change in A_{340}/minute/milligram protein) (1000), corrected for non-Ald D activity.

[c] This single ethioinine-induced liver tumor is tabulated apart from the other ethionine tumors, not only because its Ald D was more active than that of the others, but because the Ald D of this one tumor exhibited biochemical characteristics we have heretofore found only in AAF- or DAB-induced tumors (see text).

TABLE II. Aldehyde Dehydrogenase Activity in Some Other Tumors.[a]

Species	Strain	Carcinogen	Tissue	Ald D activity Number	Ald D activity Mean ± SD	Ald D activity Range
Rat	S.-D.	AAF	Lung tumor	1	9	
			Intestinal tumor	1	2	
			Salivary gland tumor	2	1 ± 1	0-1
Rat	Buffalo	MBAF[b]	Morris hepatoma 9618B	1	0	
		FPA[c]	Morris hepatoma 7777	3	3 ± 3	0-5
		FPA	Morris hepatoma 5123tc	3	11 ± 13	2-26
		FPA	Morris hepatoma 7800	3	26 ± 1	25-27
		FPA	Morris hepatoma 7794A	1	49	
Mouse	BCF_1	None	Normal liver	3	19 ± 20	5-42
		None	Liver tumor (spontaneous)	3	13 ± 17	1-32
Mastomys		None	Normal liver	1	20	
		None	Liver tumor (spontaneous)	1	0	

[a] Ald D activity is defined as in Table I legend.

[b] α-(4'methyl-)benzoylaminofluorene.

[c] *N*-2-fluorenylphthalamic acid.

ABLE III. Aldehyde Dehydrogenase Activity in Nontumorous Sprague-Dawley Rat Liver.

Liver	Number	“Tumor” isozymes[a]	Ald D activity Mean ± SD	Ald D activity Range
l adult (normal diet)	15	0	65 ± 14	39 ± 88
erating				
r postpartial hepatectomy	1	0	51	
ays postpartial hepatectomy	1	0	50	
ays postpartial hepatectomy	1	0	57	
ays postconception	3	0	3 ± 1	2-12
ays postconception	3	0	6 ± 2	5-8
al” after AAF feeding[b]				
umor macroscopically evident				
AF, then normal diet	1	0	56	
AF, then phenobarbital	2	0	85 ± 35	60-109
or elsewhere on liver				
AF, then normal diet	3	1	59 ± 5	54-63
AF, then phenobarbital	5	2	70 ± 15	53-87
AF, four cycles	5	5	57 ± 10	44-72

umor” type isozymes are defined as multiple bands appearing on PAGE gels at the appropriate location tandard conditions. Numbers given are the numbers of sections examined, and those found to contain these

ese are macroscopically and histologically normal liver sections, taken at sacrifice at least 6 months after n of a diet of AAF followed by phenobarbital, or after four cycles of AAF, or at least 12 months after start t of AAF followed by normal diet. These are the times at which grossly macroscopic tumors were first d in this experiment.

D activity is defined as in Table 1 legend.

arcinoma) showed even less activity did the normal liver. It is unfortunate the single active ethionine tumor was soelectrically focused; the tissue was y used up in routine assays and PAGE efore it was realized that it exhibited omalous Ald D activity.

ure 2 shows the same tissues, using P as coenzyme. It is clear that the al type (lower pI) isozymes, in either al liver or AAF- or DAB-induced tu-, cannot use NADP as coenzyme; the al pI isozymes characteristic of these rs used NADP approximately as effec- as NAD.

cussion. The liver tumors studied in vork were assayed and tested for iso- pattern by one individual, and evalu- histologically for degree of differentia- by another. No correlation could be l between the biochemical and the his- ical characteristics. For example, the ethionine-induced hepatocellular car- ia with a high Ald D activity had a g reaction on PAGE gels with either or NADP, while other hepatocellular iomas induced by ethionine showed low Ald D activity, faint or absent PAGE banding with NAD, and no PAGE banding with NADP. AAF-induced tumors also exhibited no correlation between biochemical and histological characteristics.

The reason for the wide quantitative variation in tumor Ald D activity is unknown. However, in the case of AAF- or DAB-induced liver tumors, the isozyme pattern we associate with tumor Ald D is invariably present, although faintly in tumors of low total Ald D activity.

This sort of faint, but recognizable tumor isozyme pattern has also been observed occasionally in the macroscopically and histologically normal sections of livers of rats that had been fed AAF many months earlier and that exhibited tumors elsewhere in the liver. In other words, the biochemical change has upon occasion been observed in areas where no histological change is yet observable. It was unexpected that the isozyme pattern found in the hepatocellular carcinomas should also be found in lesions in which hypertrophy, hyperplasia, and altered glycogen storage were the only indications of neoplasia. It was even more surprising to detect the isozyme changes in the apparently normal livers of rats treated with car-

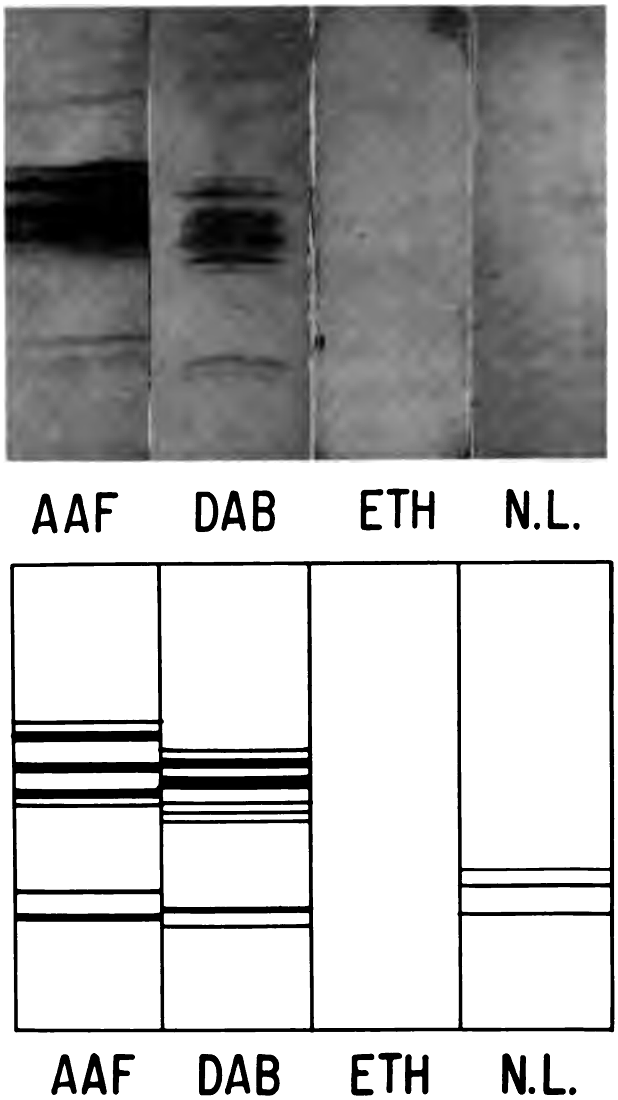

FIG. 1. Photographs and drawn counterparts of isoelectrically focused Ald D activity of rat liver tumors induced by AAF, DAB, or ethionine (ETH), or of normal rat liver (N. L.). Substrate was benzaldehyde; coenzyme was NAD. The variability of location of the AAF and DAB tumor bands is no greater than seen in a series of AAF tumors. At least part of the variation is due to slight day-to-day shifts in the location of the pH gradient. The strong bands always have a pI range of 6.9–7.2, while the weaker bands, found also in normal liver, have a pI of 6.2–6.4. Cathode is at top, anode at bottom.

cinogens. While an extensive examination was made, serial sections were not cut, and as it is not known how many altered cells have to be present for the detection of the characteristic isozyme pattern of the tumor, the interpretation of this result must be made with caution.

The role of Ald D in the economy of normal liver or of liver tumors is not clear. The enzyme has been studied by others chiefly with regard to alcohol metabolism, but ethanol is hardly to be considered an important natural metabolite. The suggestion has been made (7) that Ald D may be of importance in the metabolism of aldehydes resulting from the oxidative deamination of biogenic amines; this would seem to be a trivial consideration in the tumor econ-

The new Ald D isozymes of these s are of interest to us chiefly as a nient device for studying the mecha- whereby the carcinogenic transforma- s linked to the production of a new ular species of protein.

connection with this mechanism the cal specificity of the carcinogen is of st. Liver tumors induced with AAF or invariably show the same new Ald D es; liver tumors induced with ethio- lo not. The qualitative constancy of AF and DAB effect argues against the ility of a mutation on the structural iosynthesizing the new Ald D; on the hand, a change in the control of the expression of a structural gene for Ald D could lead to the invariant production of identical isozymes. The results for the ethionine-induced tumors suggest that there is no correlation between tumorigenesis and the alteration in the control of the structural gene for Ald D. The oncogenic property of AAF and ethionine indicates a commonality in some of the sites altered by these compounds, whereas the difference in the isozyme patterns of the Ald D suggests that, in addition, these compounds affect sites that are not in common.

The fact that the five Morris hepatomas tested—ranging from very fast to very slow growing, from poorly differentiated to well

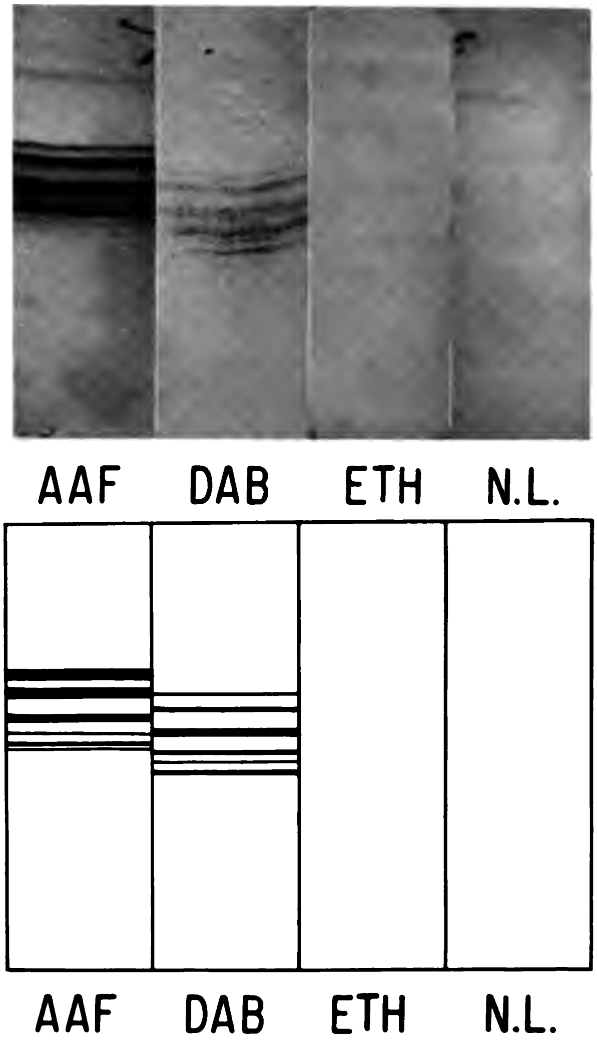

FIG. 2. Legend as for Fig. 1, except that coenzyme was NADP.

differentiated—all showed very low Ald D activity and none of the PAGE banding typical of AAF tumors, could be attributed to one of three factors: rat strain (Morris hepatomas are carried in Buffalo rats), nature of the carcinogen used, or the fact that the Morris hepatomas were all multigenerational transplants, while the other liver tumors we have studied were autochthonous. The Ald D of tumors induced (with difficulty) by feeding Buffalo rats with AAF resembles the Ald D of liver tumors in AAF-fed Sprague–Dawley rats. The critical test for choosing between the other two alternatives would be the observation of Ald D isozymes in transplanted AAF tumors; such a transplantation, however, we have not yet been able to achieve. Because of the difference in effect of ethionine, as opposed to AAF,or DAB, we feel the most likely reason for nonactivity in the Morris hepatomas tested lies in the nature of the carcinogen initially used.

The last point that we wish to emphasize is that the tumor Ald D isozymes do *not* repeat the fetal pattern. This is in contrast to Potter's suggestion (8) of "oncogeny as blocked ontogeny," and to a host of observations on other liver isozymes [see for instance (8)]. Because of this lack of recapitulation of the fetal situation, we consider the tumor Ald D production to be a possible instance of an "archeogene" (9), a gene ability repressed for untold ages and now released under the influence of certain specific stimuli.

Summary. All liver tumors induced in rats by AAF or DAB, but only one out of 29 tumors induced by ethionine, exhibited new isozymes of aldehyde dehydrogenase. These new isozymes, which differed from those of normal liver with regard to activity, pI, stability, cellular distribution, and coenzyme specificity, were not found in fetal or regenerating rat liver, nor in spontaneous liver tumors of the mouse or mastomys, nor in several other tumors appearing in rats bearing AAF-induced liver tumors, nor in any of several transplanted Morris hepatomas. The activity of the new isozymes did not correlate with histological criteria of differentiation, and the new forms were sometimes detectable in the liver of AAF-fed rats in areas where no tumor was macroscopically or histologically visible.

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Metabolic Disposition of β-Aminopropionitrile in the Rat[1, 2] (39420)

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trolled osteolathyrism, for many a laboratory curiosity, is now emerg- a clinical tool of potential importance. uccessful use of beta-aminopropioni- BAPN) in prevention of esophageal re and in overcoming the restrictive of peritendinous adhesions in animal ments (1–3) had led to human clinical Results with BAPN in treatment of ts with urethral stricture[3] and tendon ons indicate that the physical proper- scar tissue may be amenable to bio- cal control (4, 5).

hese preliminary trials, discrepancies een noted between the clinical data he predicted biochemical reactions the dose of BAPN administered. observations have raised questions ning the uptake, metabolism, and ex- of BAPN. Information in such areas contribute to safer and more effective of BAPN as well as to the interpreta- future clinical studies.

report describes a method based thin-layer chromatography (tlc) for rement of BAPN directly in urine and issues of rats. Because the major me- e of BAPN in the rat is cyanoacetic AA) (6), which is nonlathyrogenic AA was measured concurrently by a cation of the method of Sievert *et al.* Utilization of [^{14}C]nitrile-labeled made it possible to measure the rate version of BAPN to CAA by tissue enates.

rials and methods. Beta-aminopro- ile fumarate and CAA were pur- from commercial sources. Plastic precoated silica gel sheets (250 μM) without $CaSO_4$ binder (Macherey Nagel) and ninhydrin aerosol spray[4] were purchased from Brinkmann Instruments, Westbury, New York. [^{14}C]Nitrile-labeled BAPN (2.03 mCi/mmole) and [2-^{14}C]CAA (55.5 mCi/mmole) were obtained from Hoffman-LaRoche, Inc., Nutley, N.J.,[5] and ICN Isotope and Nuclear Division, Irvine, Calif., respectively. Both gave single radioactive peaks by tlc.

Female Sprague-Dawley rats weighing 200–250 g were used in groups of three for the *in vivo* studies. BAPN was diluted in sterile saline and injected ip at 400 mg/kg into one group. Diuresis was produced in a second group by administering 15 ml of saline sc prior to ip injection of BAPN. A third group was injected ip with 456 mg/kg of CAA (equivalent to BAPN dosage). Urine was collected under toluene at 2, 3, 4, 6, 12, and 48 hr, the volume was recorded, and the sample frozen at −20° until analyzed for BAPN and CAA.

Tlc of BAPN in urine. Urine samples were centrifuged and an aliquot of the supernatant from samples collected at 2, 3, 4, and 6 hr was diluted 1:10 with 80% ethanol. The 12-hr sample was diluted 1:5 with 100% ethanol. The 24- and 48-hr samples were not diluted. Two-microliter aliquots of all of the samples except the 48-hr sample were spotted on a tlc plate. The 48-hr sample required a 10-μl aliquot for assay. A BAPN standard in 80% ethanol (100 μg/ml) and control urine taken prior to administration of BAPN were cochromatographed. The tlc plate was developed in acetone, 1 M NH_4OH (9:1) for 10 cm, removed, dried in

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ented in part at the annual meeting of the Anaheim, California, April 1976 (Abstr. Fed. Proc. **35**, 679, 1976).

. Peacock, Jr., unpublished data.

[4] Ninhydrin aerosol spray (0.1% in isopropanol) from E. Merck (Catalog no. 6758).

[5] We are grateful to Hoffmann-LaRoche, Inc., of Nutley, N.J., for a supply of [^{14}C]nitrile-labeled BAPN.

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air, then heated at 90–100° for 30 min. The plate was sprayed with ninhydrin aerosol and reheated for 8–10 min at 90–100°. The violet spots corresponding to an R_f of 0.6 were scanned with a Beckman R-110 Microzone Densitometer after cutting the tlc plate into suitable strips. The tracings were cut out, weighed on an analytical balance, and the BAPN in the urine sample calculated utilizing the formula:

$$\frac{\text{experimental trace (mg)}}{\text{standard trace (mg)}} \times \text{BAPN standard spotted } (\mu\text{g}) \times \text{dilution factor} \times \frac{\text{vol of urine (ml)}}{\text{vol spotted (ml} \times 10^{-3})}.$$

CAA in Urine. Aliquots (0.1 ml) of centrifuged urine corresponding to the samples used in the BAPN study were diluted to 1.0 ml with H_2O. Sixty percent H_2SO_4 (0.2 ml) was added, followed by 10 ml of ethyl acetate (EA) saturated with H_2O. Samples were extracted for 10 min on a mechanical shaker, centrifuged at 2000 rpm for 5 min, and 5 ml of supernatant was transferred to graduated tubes containing 0.5 ml of 0.1 *M* acetate buffer at pH 5.0. The contents were shaken and the ethyl acetate evaporated under N_2 at 55–65°. All samples were brought to 10 ml with H_2O. Control specimens of urine with and without 100 μg of CAA were run concurrently as a standard and specimen blank, respectively. Aliquots of the final 10-ml dilution ranging from 0.1 to 1.0 ml were analyzed colorimetrically as described by Sievert *et al.* (7). The maximum optical density obtained against a water reference at 490 nm and corrected for the specimen blank was used to calculate the CAA content in the experimental urines according to the following formula:

$$\frac{\text{OD of experimental}}{\text{OD of standard}} \times \text{standard } (\mu\text{g}) \times \frac{10\text{ ml}}{\text{aliquot for colorimetry (ml)}} \times \frac{10\text{ ml}}{\text{EA aliquoted (ml)}} \times \frac{\text{urine vol (ml)}}{0.1\text{ ml}}$$

Characterization of the radioactivity in rat liver and brain after injection of [^{14}C]BAPN. Determination of CAA. In a preliminary experiment, the partition coefficient of [^{14}C]BAPN and [^{14}C]CAA was separately determined in 0.01 *M* unlabeled BAPN and CAA, respectively. The solutions were acidified with H_2SO_4 as in the previous determination. Ten volumes of diethyl ether previously saturated with H_2O were added and the mixture agitated for 10 min. After centrifugation, aliquots of the ether layer were added to scintillation vials containing 1 ml of 0.01 *M* BAPN and CAA in H_2O, and the ether was evaporated by an air current. Only 1 ± 0.2% (SD) of the radioactivity from [^{14}C]BAPN was found in the ether layer, while 80.0 ± 0.6% (SD) of the [^{14}C]CAA (six trials) was extracted by ether. Similar recoveries were obtained in the presence of liver and brain tissue as described below.

BAPN (8.3 mg) containing 4.95 μCi of [^{14}C]BAPN was injected iv into each of three rats. After 24 hr, the animals were sacrificed and the brain and liver were removed, blotted, and weighed. Tissue was stored at −20°. Thirty percent homogenates were prepared in 0.01 *M* $CaCl_2$. Two-tenths milliliter of liver and 0.5 ml of brain homogenate received 0.1 ml of 1% Triton X-100 in 0.01 *M* $CaCl_2$ and were brought to 1.0 ml with the $CaCl_2$ solution. After incubating for 1 hr at 37° to allow the Triton X-100 to further solubilize the tissue, the CAA present in the tissue was estimated by ether extraction. The radioactivity ascribable to ^{14}CAA in disintegrations per minute per gram of tissue was calculated by the following formula:

$$\frac{\text{net cpm}}{\text{counting efficiency}} \times \frac{10\text{ ml}}{\text{vol of ether aliquoted}} \times \text{partition ratio} \times \frac{1000\text{ mg}}{\text{wt of tissue (mg)}}.$$

The nanomoles of CAA/g equivalent to the dpm/g were calculated from the specific activity of the [^{14}C]BAPN administered.

Test for BAPN in brain tissue. One-milliliter aliquots of the 30% homogenates of ı tissue were added to 4 ml of ice-cold ıol. After 30 min in ice, samples were ·ifuged at 2000 rpm for 10 min. Four- five-tenths milliliters of supernatant reduced to less than 1.0 ml by drying :r N_2 at 60–70°. All samples were ad- d to 1.0 ml with 80% ethanol. Aliquots ml) were transferred to microvials and ı evaporated to dryness under N_2 at 60– The residues were suspended in 50 μl)% ethanol on a Vortex mixer. Radio- ity was measured by counting a 5-μl ot in 1 ml of H_2O containing 0.01 *M* ɔeled BAPN and CAA as carrier. Unla- l BAPN was added to the remaining 45 f the experimental samples to provide ιg of BAPN per 10-μl aliquot of sample ed on the tlc plate. A BAPN standard μg) in 80% ethanol was spotted also. A ; control from a rat not injected with BAPN but receiving a radioactive spike *ro* was cochromatographed. All spots ;sponding to an R_f of 0.6 were scraped ;cintillation vials; 1 ml of carrier BAPN ıdded and the samples counted.

nversion of BAPN to CAA in vitro. BAPN was added to sufficient carrier N so that 2 μ*M* contained 5 × 10^5 Labeled BAPN (20 μl) was added to ml of 0.2 *M* phosphate buffer (pH 7.4) ield concentrations of BAPN in the ; of 2.5 to 40 m*M*. Next, 20 μl of ly prepared 5% rat liver homogenate ıdded to the experimental samples. The volume of boiled homogenate was d as a control for each BAPN concen- ın. A blank containing tissue alone (no N) and tissue containing [^{14}C]CAA to ıate the recovery of the metabolite also analyzed. All samples were ɜd with O_2 for 15 sec, and screw caps tightened on the centrifuge tubes con- ıg the samples. Samples were incu- l at 37° for 30 min and 0.2 ml of 2 *N* added to terminate the reaction. One-milliliter of carrier CAA was added to *M* concentration, followed by 5 ml of yl ether equilibrated with 1 *N* HCl. s were agitated mechanically for 10 centrifuged, and 4-ml aliquots of the layer transferred to scintillation vials ıining 1.0 ml of 0.01 *M* CAA. Ether ;vaporated with an air stream at am- temperature, 10 ml of aquasol was added, and the samples were counted. Radioactivity was converted to μ*M* of CAA/g/hr by use of the specific activity value for the [^{14}C]BAPN added.

Results. Recovery of BAPN in rat urine. Figure 1 shows mean values for unchanged BAPN in the urine of three rats for 48 hr following an ip injection of 400 mg/kg of BAPN. Saline diuresis in the second group of rats produces earlier and more rapid excretion of urinary BAPN. The diuresed rats also excreted more BAPN in 24 hr than those not receiving diuresis; both groups showed negligible excretion of unchanged BAPN thereafter.

Recovery of CAA in the urine of rats given 400 mg/kg of BAPN or the equivalent dose of CAA directly. Mean values for CAA in urine of rats undergoing saline diuresis and treated with BAPN were compared with the urinary CAA found in similarly diuresed animals given the equivalent dose of CAA (456 mg/kg ip) (Fig. 2). Direct injection of CAA is followed by rapid and almost complete excretion of the compound within 48 hr. Almost 60% of directly injected CAA could be recovered contrasted to 2% recovery of BAPN-derived CAA in urine 6 hr following ip injection. Thereafter, urinary CAA excretion in animals given BAPN increased gradually over 48 hr to approximately 22% of the dose administered.

Recovery of CAA from rat liver and brain after injection of [^{14}C] BAPN. The partition study described in the Methods section showed 99% of [^{14}C]BAPN remained in the aqueous phase after extraction with diethyl ether; 80% of [^{14}C]CAA was extracted by ether under identical conditions. Radioactivity recovered by ether from rat brain and

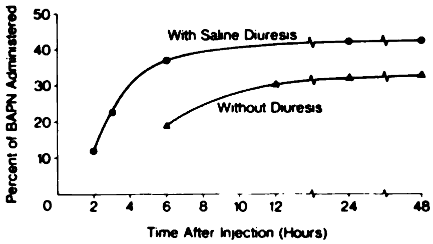

FIG. 1. Recovery of BAPN from rat urine. BAPN, 400 mg/kg, was injected into two groups of three rats each. One group received 15 ml of saline sc concurrent with BAPN dosage. The curves represent mean recoveries from each group.

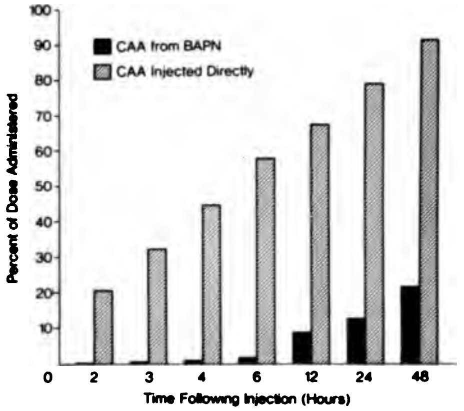

FIG. 2. Cyanoacetic acid in rat urine after injection of BAPN or cyanoacetic acid (CAA). BAPN, 400 mg/kg, or CAA, 456 mg/kg (dosage equivalent to BAPN), was injected ip into two groups of three rats given 15 ml of saline sc concurrent with drug dosage. The histograms represent mean recoveries from each group.

liver in animals injected with $[^{14}C]$BAPN and sacrificed 24 hr later is therefore taken as $[^{14}C]$CAA (Table I).

When the aqueous phase remaining from the experimental samples of rat liver was extracted with ether a second time, 80% of the radioactivity again distributed itself into the ether phase, suggesting that the BAPN-derived radioactivity remaining in the aqueous phase after the first extraction was also CAA.

Tlc for BAPN in brain tissue after iv injection of $[^{14}C]$BAPN into rats. An aliquot of tissue homogenate prepared from brains of rats sacrificed 24 hr after iv injection with $[^{14}C]$BAPN was analyzed for BAPN as described. Calculations from Table II show that 76% of the radioactivity added as $[^{14}C]$BAPN to the control and measured prior to chromatography could be recovered from the spot corresponding to BAPN (R_f = 0.6) after chromatography. Experimental tissues yielded no radioactivity in the corresponding spots from the unlabeled carrier BAPN added (Table II).

Conversion of $[^{14}C]$BAPN to CAA by rat liver homogenate in vitro. Conversion of $[^{14}C]$BAPN to $[^{14}C]$CAA appears approximately linear in the range of 2.5 to 10 m*M* inclusive of $[^{14}C]$BAPN added (Table III). The data indicate that a small percentage of BAPN added was changed to CAA. Table III also shows that under the conditions of this experiment addition of a starting concentration of 40 m*M* BAPN resulted in formation of a lesser amount of CAA than that obtained from a 30 m*M* BAPN concentration.

Discussion. Results of these experiments indicate that active half life of BAPN, administered to rats as a single ip dose, is short. Urinary excretion of unchanged BAPN began shortly after injection and approached maximum within 12 hr at which time approximately 33% of the administered dose was excreted (Fig. 1). This effect was exaggerated by saline diuresis, presumably by increasing renal excretion before BAPN could be deposited in tissues. Negligible BAPN was recovered in urine after 24 hr in both nondiuresed and diuresed rats (Fig. 1). The radioactivity in rat brain and

TABLE I. RECOVERY OF CAA FROM RAT LIVER AND BRAIN AFTER INTRAVENOUS INJECTION OF $[^{14}C]$BAPN.[a]

Tissue		$[^{14}C]$CAA Recovered[b]	CAA Recovered (nmoles/g)
A. Liver			
Control (no BAPN)		17	–
Experimental:	A	14,062	152
	B	8,872	96
	C	10,453	113
B. Brain			
Control (no BAPN)		15	–
Experimental:	A	7,894	85
	B	6,733	73
	C	7,198	78

[a] 8.3 mg of BAPN containing 4.95 μCi were injected. 24 × 10^3 nmole = 1 μCi (2.22 × 10^6 dpm).

[b] Corrected for 95% recovery of $[^{14}C]$CAA added to liver tissue from control rats (no BAPN).

TABLE II. EFFECT OF TLC ON THE RADIOACTIVITY IN BRAIN HOMOGENATES OF RATS INJECTED WITH $[^{14}C]$BAPN.[a]

Sample	Radioactivity (dpm/g) Before tlc	Radioactivity (dpm/g) After tlc
Control + $[^{14}C]$BAPN[b]	1925	1472
Experimental, Rat no. 1	7105	0
Rat no. 2	4822	0
Rat no. 3	7002	0

[a] 8.3 mg of BAPN + $[^{14}C]$BAPN (4.95 μCi) were injected and the animal sacrificed 24 h later.

[b] Control brain homogenate received 3360 dpm of $[^{14}C]$BAPN and was analyzed and cochromatographed along with the experimental brain tissues.

E III. CONVERSION OF [^{14}C]BAPN TO CAA BY RAT LIVER HOMOGENATE[a] *in vitro*.

[^{14}C] CAA[b] (dpm)	[^{14}C] BAPN[c] Initial concentration (m*M*)	Ether layer (A-B) (dpm)	μM CAA/g/hr
–	–	15	–
5875	–	4870	–
–	2.5	140	1.12
–	5.0	296	2.37
–	10.0	570	4.56
–	20.0	702	5.62
–	30.0	992	7.94
–	40.0	886	7.09

nty microliters of fresh (A) or boiled (B) 5% homogenate equivalent to 1 mg of tissue was d with the concentrations of [^{14}C]BAPN listed r 30 min in 0.2 ml of 0.2 *M* phosphate buffer.

partition ratio of 4870 dpm in the ether layer e 5875 dpm added as [^{14}C]CAA provided a n factor for the dpm obtained with experimen- les.

10^5 dpm of [^{14}C]BAPN = 2 μmole of the nd.

n animals sacrificed 24 hr after injec- f [^{14}C]BAPN appeared to be exclu- nonlathyrogenic [^{14}C]CAA (Tables I). Although based on a limited num- animals, the observations in the urine ssues suggest that concentrations of could have diminished within 24 hr els that were not pharmacologically ve.

retion of BAPN-derived CAA in the occurred over a longer time course, ring several hours after injection and sing slowly and gradually throughout -hr study period (Fig. 2). An equiva- ose of CAA was promptly excreted 2). Because the pK of CAA is 2.45 t physiological pH, directly injected would exist as a polar anion suscepti- glomerular filtration (10). The rapid ion of directly injected CAA (Fig. 2) sistent with this mechanism. Slower ion of BAPN-derived CAA supports pothesis that unexcreted BAPN is se- red in tissues where it is stored and uently metabolised to CAA before slowly released. This hypothesis is hened further by the finding that 24 r an injection of isotopically labeled virtually all radioactivity extracted at brain and liver was present as [^{14}C]CAA (Tables I and II). Similar findings were reported by Waddel *et al.* (11).

The work of Haney *et al.* (12) also points to the transient existence of the biological activity of BAPN *in vivo*. Single daily doses of BAPN (23 mg/kg) for 10 days did not inhibit totally collagen cross-linking because an additive effect was obtained by administering D-penicillamine. These data suggest that renewed lysyl oxidase activity (13) and collagen cross-linking had time to occur between BAPN doses.

The teratological properties of BAPN (14, 15) in the rat show similar pharmacokinetic limitations. Pratt and King (15) demonstrated a close correlation between cleft palate induction and inhibition of collagen cross-linking when BAPN was administered to pregnant rats on Day 15 of gestation but not on Day 14. Our data (Fig. 1; Table II) are in accord with the notion that BAPN may decrease in both maternal and fetal tissues within 24 hr following dosage on Day 14 to levels below those required to produce this abnormality in the fetus.

Although the major location, rate, and mechanism of BAPN degradation are not known, the *in situ* conversion of retained BAPN to CAA strongly suggests a metabolic system presumably including monoamine oxidase found in a number of tissues. Data reported in this paper substantiate the relatively slow kinetics of this system in the rat (Table III). Earlier findings of Sievert *et al.* (8) also show enzymatic conversion of BAPN to CAA *in vitro* by rat liver homogenate to occur more slowly than with similar preparations from seven other species. In the radiometric assay, with [^{14}C]BAPN made to contain 2.5 × 10^5 cpm/μm the enzyme activity of 1 mg of rat liver homogenate could be readily measured and the percentage conversion of BAPN to CAA was extremely small (Table III). Such observations are in agreement with the small conversion of BAPN to CAA noted in isolated rat livers profused with BAPN.[6] It is of interest that an initial concentration of 40 m*M* BAPN added as substrate to rat liver homogenate (Table III) showed inhibition analogous to that reported by Page and

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[6] J. H. Fleisher, K. Brendel, M. Chvapil, and E. E. Peacock, Jr., submitted for publication.

Benditt (16) with pig plasma amine oxidase and kynuramine as substrate.

Because oxidative deamination appears to be the rate-limiting step in the ultimate metabolic disposition of BAPN in rats, unpredicted dose-response patterns following chronic administration of BAPN may be due to "overloading" of the degradative pathway, with storage of active BAPN and prolongation of lathyritic effect. Such data may explain prolonged elevation of collagen a:B ratios observed by Bentley *et al.* (17) after 17 days of BAPN administration. Interference with drug degradation by a specific monoamine oxidase inhibitor also might be expected to enhance osteolathyritic effect of BAPN in the rat (18).

As yet, detailed studies of BAPN metabolism in man have not been carried out. However, amine oxidase systems demonstrate enormous species variability in kinetics, substrate specificity, and cofactor requirements. In addition, the potential role of plasma amine oxidase, present in man but not in the rat, has not been explored with respect to BAPN metabolism. Further studies will be necessary to delineate the pharmacologic parameters for controlled lathyrism in humans.

Summary. Enzymatic conversion of the lathyrogen, β-aminopropionitrile (BAPN) to the deaminated, nonlathyrogenic metabolite, cyanoacetic acid (CAA) by rat liver homogenates was found to be less than 1%. Urine of rats injected with 400 mg/kg of BAPN ip contained 32% unchanged BAPN in 24 hr, with negligible recovery thereafter. BAPN-derived CAA showed 12 and 22% recovery in the urine in 24 and 48 hr, respectively. These data suggest that significant amounts of unchanged BAPN may not exist in rat tissues after 24 hr, and that BAPN-derived CAA in such tissues is gradually excreted. The observation that radioactivity in rat brain and liver 24 hr after [^{14}C]BAPN injection is present exclusively as [^{14}C]CAA supports this interpretation.

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Determination of Glucose Utilization in the Dog with [2-^{3}H], [6-^{3}H]-, and [U-^{14}C]Glucose[1] (39421)

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ucose labeled with ^{14}C has been widely
as a tracer for the study of *in vivo*
se metabolism in various species. The
cation of [U-^{14}C]glucose kinetics to
se turnover studies is, however, com-
ed by the recycling of carbon which
ly limits the interpretation of the ex-
iental data (1-6). An alternative ap-
:h, based on the use of [^{3}H]glucose, has
proposed in order to minimize this
em (4, 6-9). The use of tritiated glu-
as a tracer of glucose metabolism has
investigated in various species (3, 4, 7-
The loss of tritium generally exceeds
ss of ^{14}C from circulating glucose, sug-
g less recycling of tritium than of ^{14}C
-9). Formation of water is the predomi-
early fate of tritium from metabolized
se (7, 9) and the dilution of tritium
glucose in a large pool of body water
nizes the recycling of this tracer to the
se pool.

^{3}H]Glucose and [6-^{3}H]glucose have
used most frequently as ^{3}H-labeled
s for estimating parameters of glucose
olism (7-11, 13-16). Their use in
ination with [U-^{14}C]glucose have al-
l various investigators to estimate the
e of glucose-carbon recycling in intact
ls (9, 18). Only a limited number of
s have been conducted which compare
ss of tritium from [2-^{3}H]glucose and
]glucose in the same experiment. Such
s would allow one to estimate the rate
icose utilization as well as to obtain
information on the extent of operation
itile cycles."

purpose of this study was to determine the rate of glucose utilization in the dog using [2-^{3}H]- and [6-^{3}H]glucose in combination with [U-^{14}C]glucose.

Materials and methods. Experimental animals and diet. One-year-old female beagles[2] were housed in individual pens with raised floors and were fed a maintenance level of a commercial diet.[3] The diet contained 36, 40, and 24% of metabolizable calories from protein, fat, and carbohydrate, respectively. Water was available *ad libitum*. Lights in the temperature-controlled rooms were on from 7:00 AM to 7:00 PM and off from 7:00 PM to 7:00 AM. At the time of the experiment, the dogs weighed approximately 8 to 10 kg.

Turnover study. Glucose turnover studies were performed on seven dogs fasted for 48 hr. One hour prior to the experiment, indwelling catheters were inserted in the jugular vein of unanesthetized dogs. Teflon catheter needles[4] were used to eliminate surgery during the implantation. Dogs were in their pens and allowed access to water during the experiment. Four dogs were injected with [2-^{3}H]glucose[5] (250 μCi) and [U-^{14}C]glucose[5] (20 μCi) mixture in 5.0 ml of 0.9% NaCl. Simultaneous administration of [6-^{3}H]glucose[5] (250 μCi) and [U-^{14}C]glucose (20 μCi) was performed in the other three dogs. Blood samples (5.0 ml) were withdrawn at timed intervals up to 5 hr. The initial samples were taken at 3-min intervals for the first 15 min, and at 5 min intervals for the next 15 min. Subsequent blood samples were taken at 15-min intervals up to 1 hr, and then at 30-min intervals thereafter. Blood samples were collected in

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[2] Laboratory Research Enterprises, Inc., Kalamazoo, Mich.

[3] Ken-L-Ration, The Quaker Oats Company, Chicago, Ill.

[4] Becton, Dickinson & Co., Rutherford, N.J.

[5] Amersham/Searle Corporation, Arlington Heights, Ill.

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chilled tubes containing heparin and sodium fluoride. Dogs were handled gently and did not appear excited during sampling. The total volume of blood withdrawn per dog per day was approximately 90 ml. After 1 week dogs previously injected with [2-^{3}H]glucose were administered with [6-^{3}H]glucose and vice versa.

Glucose and glucose-specific radioactivity determinations. Plasma was promptly separated and deproteinized with barium hydroxide and zinc sulfate. Glucose was isolated by passing an aliquot of the deproteinized plasma through an ion exchange column (9, 17). Determination of glucose-specific radioactivity by paper chromatography verified that the column separated [U-^{14}C]glucose from its ^{14}C-labeled metabolites. Further, results from a preliminary experiment showed that recovery of labeled glucose after passage through the ion exchange column averaged 95%. The column eluate and washings were lyophilized and taken up in a small volume of water. Scintillation cocktail[6] was added and the radioactivity in the sample was quantitated by liquid scintillation spectrophotometry. Tritium counts were corrected for spillover of ^{14}C-counts. The counting efficiency was obtained with an external standard. Plasma glucose concentration was determined in other aliquots with glucose oxidase.[7] Tritium and ^{14}C-specific radioactivity of glucose was then calculated.

Calculations. Parameters of glucose metabolism estimated by single injection of labeled glucose were calculated using both graphical and exponential analysis. The linear plot of glucose specific radioactivity versus time was constructed. The glucose replacement rate, transit time, total body glucose mass, glucose-carbon recycling, and glucose mass of sampling pool were then calculated graphically according to Katz *et al.* (6, 9). The log of specific radioactivity versus time curve of glucose was also constructed. The curve obtained was fitted into a two-component exponential equation. Estimates of the coefficient and exponent of each component were obtained by a curve "peeling" process. The terminal exponential of the curve was fitted by least square procedure, and then, by subtraction of this line from the earlier part of the curve, a second curve was obtained. Glucose replacement rate and total body glucose mass in the dog were then calculated using the two-component exponential equation.

Statistical analyses. The data were treated statistically by Student's *t* test (19).

Results. Plasma glucose levels were relatively constant throughout the experimental period. The coefficient of variation of glucose concentration (14–19 samples) was less than 7%. Body weight and plasma glucose levels in dogs injected with [2-^{3}H]glucose were not significantly different from those in dogs administered [6-^{3}H]glucose (Table I).

Figure 1 presents a linear plot and Fig. 2 a semilogarithmic plot of plasma glucose radioactivity versus time after a single injection of [2-^{3}H]- or [6-^{3}H]glucose. These curves show an initial rapid decline followed after about 20 to 30 min by a curvilinear change up to 5 hr postinjection. From the areas under these curves (Fig. 1), parameters of glucose metabolism were calculated by the graphical method of Katz *et al.* (6, 9). In Fig. 2 the straight lines represent exponential functions that give the line of the best fit for the decay curves. These curves were represented by an exponential equation with two terms.

Estimates of glucose metabolism parameters in dogs fasted for 48 hr are presented in Table I. The glucose replacement rate using the graphical method of analysis averaged 4.6 mg/min/kg body weight for [2-^{3}H]glucose. This value is similar to values reported by others (13, 15). The glucose replacement rate calculated by graphical or by exponential analysis was higher when [2-^{3}H]glucose rather than [6-^{3}H]glucose was utilized as tracer. However, values obtained by exponential analysis were more variable and thus differences between [2-^{3}H]glucose and [6-^{3}H]glucose were statistically different only when the graphical analysis was utilized. Values for the rate of glucose utilization obtained by exponential analysis were also slightly higher than values obtained by graphical analysis (Table I).

Estimates of glucose-carbon recycling

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[6] 3a70, Research Products International Corporation, Elk Grove Village, Ill.

[7] Glucostat T. M., Worthington Biochemical Corp., Freehold, N.J.

TABLE I. GLUCOSE METABOLISM IN FASTED DOGS ESTIMATED BY USING A SINGLE INJECTION OF MIXTURES OF [2-^{3}H]GLUCOSE OR [6-^{3}H]GLUCOSE AND [U-^{14}C]GLUCOSE.

	Tritiated tracer	
	[2-^{3}H]glucose	[6-^{3}H]glucose
Body weight (kg)	9.42 ± 0.28[a]	9.43 ± 0.30
Plasma glucose (mg/100 ml)	102 ± 2	97 ± 5
Glucose replacement rate		
[^{3}H]glucose (mg/min/kg)	4.6 ± 0.2 (5.3 ± 0.9)	3.7 ± 0.2[b] (3.9 ± 0.4)
[^{14}C]glucose (mg/min/kg)	3.2 ± 0.2	3.1 ± 0.1
Glucose-carbon recylcing (%)	31 ± 2	14 ± 2[b]
Glucose transit time (min) ^{3}H[c]	67 ± 3	76 ± 4
^{14}C[c]	91 ± 3	89 ± 5
Glucose body-mass (mg/kg) ^{3}H	308 ± 15	280 ± 18
^{3}H	(261 ± 15)	(225 ± 17)
^{14}C	284 ± 18	290 ± 21
Glucose mass of sampling pool (mg/kg) ^{3}H	210 ± 16	199 ± 15

[a] Results expressed as mean ± SE for seven dogs. Numbers in parenthesis indicate values calculated by exponential analysis.

[b] [2-^{3}H]Glucose versus [6-^{3}H]glucose, significantly different ($P < 0.05$).

[c] Indicates tracer utilized in the calculations.

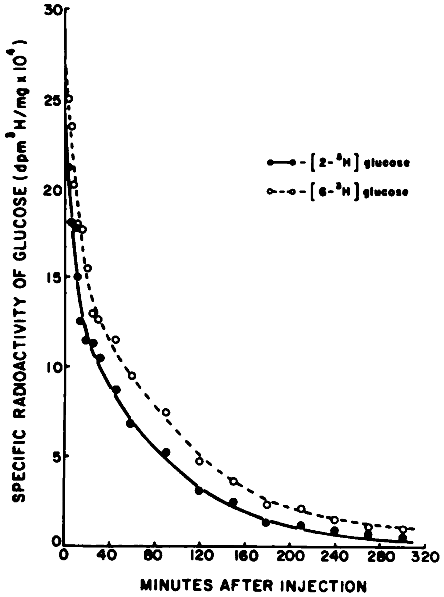

FIG. 1. A linear plot of glucose-specific radioactivity and time after single injection of [2-^{3}H]- or [6-^{3}H]glucose in a dog fasted for 48 hr.

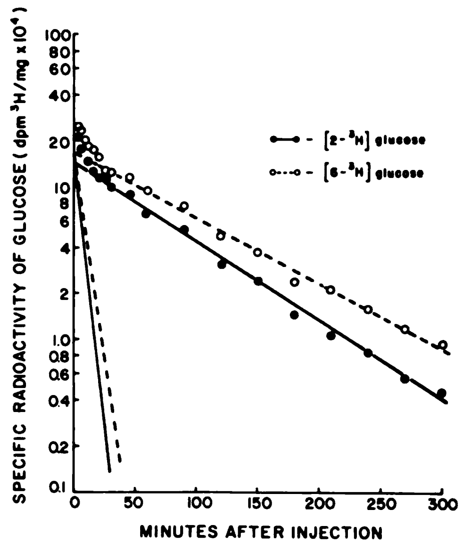

FIG. 2. A semilogarithmic plot of glucose-specific radioactivity and time after single injection of [2-^{3}H]- or [6-^{3}H]glucose in a dog fasted for 48 hr.

were obtained from the glucose replacement rates of [2-^{3}H]- or [6-^{3}H]glucose and [U-^{14}C]glucose. Glucose-carbon recycling estimated with [2-^{3}H]glucose in fasted dogs averaged 31% (Table I). This value compares favorably with a previous report (13). The degree of glucose-carbon recycling obtained with [2-^{3}H]glucose was significantly higher than when [6-^{3}H]glucose was utilized (Table I).

The time which a glucose molecule is present in the body from the time of synthesis to the time of its catabolism or loss (transit time) was also estimated using [2-^{3}H]-, [6-^{3}H]-, or [U-^{14}C]glucose as tracers. The glucose transit time obtained with [6-^{3}H]glucose was 76 min; this value was 12% longer than that obtained with [2-^{3}H]glucose. However, the difference in transit time between the two tracers was not statistically significant (Table I). Estimates of transit time obtained with ^{14}C as tracer averaged approximately 90 min.

The total body mass of glucose in fasted dogs determined by graphical analysis averaged 308 ± 15 and 280 ± 18 mg/kg body weight with [2-^{3}H]- and [6-^{3}H]glucose, respectively. These values were in close agreement with the estimates of body mass obtained with [^{14}C]glucose (Table I). Values obtained by exponential analysis were relatively lower but not significantly different from those obtained by graphical analysis. There were no significant differences in total body glucose mass obtained with [2-^{3}H]- or with [6-^{3}H]glucose. The glucose mass of the sampling or rapidly mixing pool was determined from the zero-time intercept of the glucose specific activity curves. Estimates of glucose mass of the sampling pool were also similar whether [2-^{3}H]glucose or [6-^{3}H]glucose was utilized as tracer.

Discussion. In the present study the *in vivo* metabolism of [2-^{3}H]glucose and [6-^{3}H]glucose was compared in dogs fasted for 48 hr. The rate of glucose utilization estimated with [6-^{3}H]glucose was about 20% lower than when [2-^{3}H]glucose was utilized as the tracer. This indicates that the loss of tritium from [2-^{3}H]glucose exceeds the loss of tritium from [6-^{3}H]glucose in the dog. The *in vivo* detritiation of [2-^{3}H]glucose has also been reported to be faster in rabbits (18), sheep (16), mice (20), and chickens (17) than the detritiation of [6-^{3}H]glucose. Recent reports suggest that this greater loss of tritium from [2-^{3}H]glucose than from [6-^{3}H]glucose is due to the operation of a "futile" cycle between glucose and glucose-6-phosphate (18, 21). It thus appears that significant recycling between glucose and glucose-6-phosphate is probably occurring in the dog and that our estimates of glucose utilization obtained with [2-^{3}H]glucose (Table I) represent the total rate of glucose utilization including glucose recycling at the hexokinase-glucose phosphatase level.

Simultaneous administration of tritiated and [U-^{14}C]glucose has been used to estimate glucose-carbon recycling, i.e., reappearance of ^{14}C-labeled three-carbon fragments in newly synthesized glucose. In the present study the extent of glucose-carbon recycling estimated with [6-^{3}H]- and [U-^{14}C]glucose was 55% lower than when [2-^{3}H]- and [U-^{14}C]glucose was utilized. Since the degree of glucose-carbon recycling is obtained from the difference between the [^{3}H]- and [^{14}C]glucose replacement rates, the higher estimates obtained with [2-^{3}H]glucose than with [6-^{3}H]glucose further suggest the presence of a "futile" cycle between glucose and glucose-6-phosphate in the dog. Altszuler *et al.* (22) have also recently reported that glucose turnover using [2-^{3}H]glucose was higher than when [6-^{14}C]glucose was used. Our estimates of the magnitude of this "futile" cycle depend in part on how fast glucose molecules subjected to this cycle in gluconeogenic organs of the dog equilibrate with the plasma pool relative to the rapidity with which three-carbon intermediates are formed. Estimates of the relative rates of these two processes were not obtained in the present study.

With graphical method of analysis, our estimate of the total glucose mass averaged 308 ± 15 and 280 ± 18 mg/kg with [2-^{3}H]glucose and [6-^{3}H]glucose, respectively. Our estimates compare with the reported values obtained with [2-^{3}H]glucose for rats (260 mg/kg) (9), rabbits (290 mg/kg) (9), or dogs (290 mg/kg) (23). Estimates of the glucose mass of the sampling or rapidly mixing pool are only rough approximations. As discussed by Katz *et al.* (9) due to the rapid change in slope of the initial curve (Fig. 1), these estimates are subject to considerable error.

Exponential analysis of the data has been extensively used in studies with labeled glucose. In the present study comparisons between the graphical and exponential methods of analysis were made. Theoretically, these two methods of analysis should provide similar results. Comparable results were obtained for glucose replacement rate and total body glucose mass; however, the values obtained with the exponential analysis were more variable than with the graphical method. Possibly the data would have been better represented if we had considered models containing more than two glucose pools.

Summary. Parameters of *in vivo* glucose metabolism in adult female dogs fasted for 48 hr were estimated after a single injection of [2-^{3}H]- or [6-^{3}H]glucose in combination with [U-^{14}C]glucose. The glucose replacement rate estimated with [2-^{3}H]glucose averaged 4.6 mg/min/kg and was about 25% higher than the values obtained with [6-H]glucose. Estimates of glucose-carbon recycling were more than twice as high (31 vs 4%) when [2-^{3}H]glucose was utilized as racer than when [6-^{3}H]glucose was utilized. These differences suggest the presence of a "futile" cycle between glucose and glucose-6-phosphate in the dog.

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Lung Cyclic AMP: Selective Decrease with Hypoxia (39422)

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Recent evidence suggests that adenosine 3′,5′-monophosphate (cAMP) and guanosine 3′,5′-monophosphate (cGMP) levels are potentially important in the regulation of lipid metabolism, contraction of smooth muscle in blood vessels and airways, and tissue growth (1–3). Since hypoxia is known to affect all of these parameters (4–6), we investigated the alterations in cyclic nucleotide levels in lung following hypoxic exposure. Cyclic nucleotides were also examined in liver tissue to evaluate comparatively with lung, and with fasting, since food intake is substantially reduced in rats during hypoxic exposures (7).

Methods. Male Long Evans Hooded rats weighing 250–300 g were exposed to 24-hr hypobaric hypoxia in an altitude chamber at 7193 m (23,600 ft: p_B = 280 mm Hg). Rate of ascent and descent was 1000 ft·min^{-1}. The level of hypoxia was chosen to simulate clinical and altitude conditions as well as to correlate with previous experiments on the effects of hypoxia, performed in this laboratory (8). All animals received water *ad libitum* and the hypoxic group was food deprived for the 24-hr period. At the appropriate times animals were removed from the altitude chamber and killed within 15 min by decapitation. Tissues were quickly removed, freeze clamped in liquid nitrogen, and stored at −76° until assayed. Forty milligrams of the frozen tissue were homogenized in 2 ml of 6% trichloroacetic acid, centrifuged (2000 *g*), and the supernatant extracted with ethyl ether saturated with water. The aqueous portion was dried under nitrogen on a 60–70° steam bath, resuspended in 2–4 ml of 0.05 *M* sodium acetate (pH 6.2), and assayed for cyclic nucleotides using radioimmunoassay kits (9). Statistical analyses were carried out using Student's *t* tests (10).

Results and discussion. No differences in lung dry:wet ratios were observed between hypoxic exposed animals and controls, indicating that little edema was present following acute hypoxic stress in rat lung. Lung and liver cyclic nucleotide levels are shown in Table I. In contrast to liver (fed *ad libitum* condition), lung contains higher endogenous cAMP and cGMP levels. Acute hypoxia resulted in a significant ($P < 0.025$) decrease in cAMP while liver cAMP was unaltered. Cyclic GMP for both tissues was not affected by hypoxia. These data indicate that acute hypoxic stress has a selective action in decreasing lung cAMP.

Although a 24-hr fast did not markedly alter cyclic nucleotide levels, a 72-hr fast resulted in a threefold increase in lung cAMP while liver cAMP increased eightfold. In both liver and lung, cGMP was unaltered. Although not shown, lung glycogen was unaffected by the 72-hr fast, whereas liver showed a 40-fold decrease in glycogen content, consistent with early studies (11, 8). These data emphasize the importance of the nutritional status of the animal on endogenous cyclic nucleotide levels and indicate that the preferential decrease in cAMP seen with hypoxic exposure cannot be explained as an effect due to anorexia. It is interesting to note that epinephrine will increase cAMP levels and that any stress, including starvation, may increase epinephrine and lead to elevated levels of cAMP. Thus, it is not clear at this time if the stress of food deprivation is the critical factor in stimulating cAMP levels. Nevertheless, nutritional stress appears to have a separate and distinct action on cAMP than seen with hypobaric hypoxic stress.

The association of decreased cAMP levels or decreased cAMP–cGMP ratios with vasoconstriction and tissue growth indicate that alterations of cyclic nucleotide levels may be of functional significance in the hypoxic lung. Constriction of vascular smooth muscle associated with a decrease in cAMP/cGMP ratio has been reported by others (12). Hypoxia is known to cause pulmonary

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TABLE I. CYCLIC NUCLEOTIDE LEVELS IN LUNG AND LIVER.[a]

	pmoles/mg dry weight			
Cyclic nucleotide	Control (Fed *ad libitum*)	Hypoxia (24 hr)	Fasted (24 hr)	Fasted (72 hr)
cAMP				
Lung	22.96 ± 2.63	8.24 ± 0.93*	26.74 ± 4.93	77.03 ± 14.00*
	(*n* = 6)	(*n* = 7)	(*n* = 10)	(*n* = 4)
Liver	4.70 ± 1.47	5.98 ± 2.06	6.43 ± 2.28	37.68 ± 13.70*
	(*n* = 6)	(*n* = 4)	(*n* = 4)	(*n* = 4)
cGMP				
Lung	3.01 ± 0.48	2.53 ± 0.36	2.00 ± 0.29	2.90 ± 1.13
	(*n* = 3)	(*n* = 7)	(*n* = 9)	(*n* = 4)
Liver	0.26 ± 0.06	0.39 ± 0.16	0.26 ± 0.06	0.26 ± 0.06
	(*n* = 3)	(*n* = 4)	(*n* = 4)	(*n* = 4)
$\frac{\text{cAMP}}{\text{cGMP}}$				
Lung	7.62	3.25	13.37	26.56
Liver	18.07	15.33	24.73	144.92

[a] Values are mean ± SE. Hypoxic animals were deprived of food and exposed to a simulated altitude of 7193 m.

* Statistically significant from controls ($P < 0.025$).

vasoconstriction. This raises the question of whether the decrease in cAMP observed in our investigation may serve as an underlying mechanism for the hypoxic pressor response (increase in mean pulmonary arterial pressure). Somewhat surprising was that cGMP was not elevated with hypoxia since prostaglandin (PG) $F_{2\alpha}$-like substances have been shown to be released with hypoxia (13), and Vaughan and co-workers (14) have shown that cGMP plays an important role in regulating PG synthesis and release. Whether cGMP is linked to PG $F_{2\alpha}$ in lung remains to be determined.

An additional observation is that decreased levels of cAMP have been associated with tissue growth and elevation of cAMP associated with its suppression (15). In lung, where growth is increased with hypoxia, there is a decrease in cAMP, whereas in liver in which no such growth occurs, the cAMP levels are unchanged.

Since the lung is a heterogeneous organ comprised of at least 38 different cell types it should be noted that changes in tissue cyclic nucleotide levels may not totally reflect the response of any particular function or any specific cell type. Although the present study does not delineate the functional role of lung cyclic nucleotides it does provide fundamental information concerning changes in their levels during altered physiologic states.

Summary. Rats, food-deprived and exposed to hypobaric hypoxia (7193 m or 23,600 ft) for 24 hr showed a significant decrease in lung adenosine 3′,5′-monophosphate (cAMP). Lung guanosine 3′,5′-monophosphate (cGMP) was unaltered as well as liver cAMP and cGMP. In contrast, rats fasted for 72 hr showed a significant threefold increase in lung cAMP and eightfold increase in liver cAMP. Endogenous cGMP for both tissues was unchanged by a 72-hr fast. These data indicate that acute hypoxic stress has a selective action in decreasing lung cAMP and that this effect is not related to anorexia.

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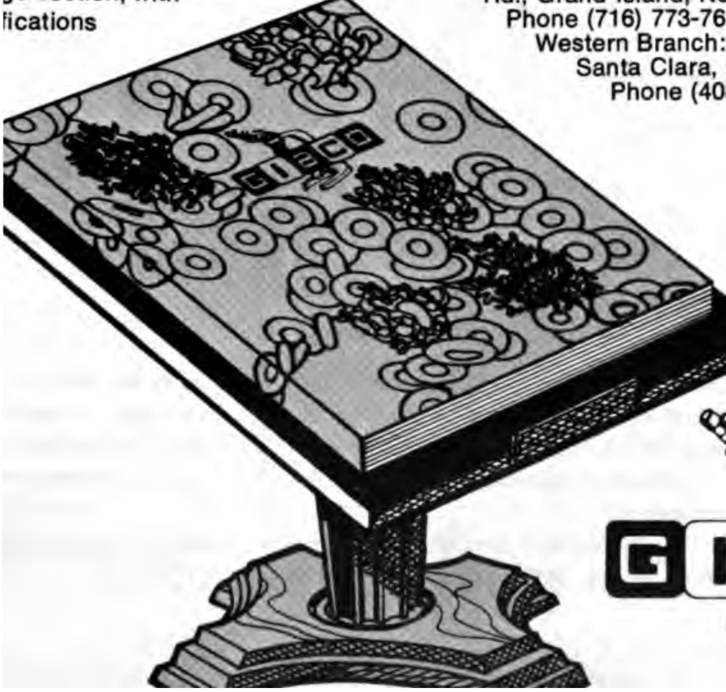

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ects of Ozone on the Respiratory Epithelium and Alveolar Macrophages of Mice. I. Interferon Production[1] (39423)

DUL L. IBRAHIM, YUAN CHUNG ZEE, AND JOHN W. OSEBOLD

Department of Veterinary Microbiology, University of California, Davis, California 95616

'fects of inhaled ozone on the func-
:he respiratory tract are of continu-
est due to the increasing importance
r pollutant. Prolonged and repeated
: of mice to ozone was found to
:eration in the synthesis of DNA,
nd protein by lung tissue, together
liferative and degenerative changes
'acheobronchial mucosa (1, 2). It
rved by Miller and Ehrlich (3) that
exposed to high levels of ozone be-
ghly susceptible to infections with
ed bacteria.

:ron is a protein synthesized and
by cells when induced by viruses or
eotides; the molecule has a wide
ı of antiviral activity (4). Prepara-
nterferon have been used in various
experiments to study resistance
ooth local and systemic virus infec-
erigan *et al.* (5) reported that the
influenza virus infection would be
when interferon was sprayed onto
r respiratory tract. Respiratory dis-
owing rhinovirus exposure was pre-
y the procedure. In other studies
n inducers, both viral and nonviral,
n used to stimulate the endogenous
on of interferon. However, infor-
s needed regarding the effects of
ı interferon production both *in vitro*
vo. Interferon release by lung cells
ne exposed mice was studied here
it is a sensitive indicator of altered
function and the release of this
also has implications for resistance
ost to respiratory viral infections.
acity of the respiratory epithelium
olar macrophages to produce inter-
vitro is shown. In addition, evi-
an inhibitory effect on interferon synthesis by respiratory epithelium following *in vivo* exposure to ozone is presented.

Materials and Methods. Viruses. The California 11914 strain of Newcastle disease virus (NDV) was used as an interferon inducer. The Indiana strain of vesicular stomatitis virus (VSV) was employed in interferon assays.

Polynucleotide. Poly(I:C) (inosine:cytidine) was purchased from Biopolymers, Chagrin Falls, Ohio.

Mice. Ten week old specific pathogen-free male mice of the Swiss Webster strain (Hilltop Lab. Animals, Inc., Scottsdale, Penn.) were used. The mice were housed with cage-filter tops in an isolated room with 14 air exchanges per hour at a temperature of approximately 23°.

Ozone exposure. Mice were exposed to ozone according to the method of Plopper *et al.* (6). The mice were housed in an ozone chamber with 30 air exchanges per hour and exposed for 14 or 21 days at a 0.8 ppm concentration of ozone. At the end of the exposure period the mice were removed to ambient air and kept there for another 24 or 10 days. Control mice were held in a similar chamber and exposed to ambient air. Air was introduced into ozone chambers and ambient chambers through a CBR (chemical, bacteriological, radiological) filter.

Organ cultures. Organ cultures were prepared according to the technique described by Hoorn and Tyrrell (7). Tracheal explants from two mice were placed in a sterile 35 mm × 10 mm plastic culture dish to which 5 ml of maintenance medium (medium 199) containing 50 µg/ml gentamicin and 0.3% bovine serum albumin was added. The cultures were incubated at 35° in a humid atmosphere of 5% CO_2 in air.

Macrophage cultures. Alveolar macrophages were obtained from the mice using a slight modification of the technique described by Medin *et al.* (8). Lung lavages

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ved.

from 18 mice were pooled and the cells were sedimented from lavage fluid by centrifugation at 1000 rpm for 30 min at 5°. The supernatant was decanted and the cells were resuspended in 4 ml of MEM containing 10% fetal calf serum (FCS) and 50 μg/ml gentamicin. The cell suspension was then dispensed equally into 10 × 35-mm plastic culture dishes in 1-ml amounts. The macrophages were given time to adhere to the plastic surface during a 2-hr period at 37° in a 5% CO_2 atmosphere. Unattached cells were removed by aspirating the medium and the adherent cells were washed three times with 2 ml of Hank's solution. Average cell counting from 20 petri dishes indicated that the culture dish contained approximately 4.0×10^5 (2.5-5.0×10^5) cells.

Interferon production in organ culture. Organ cultures were inoculated with interferon inducers on the fourth hour of incubation. The medium was removed and 0.2 ml of NDV suspension containing 5×10^5 plaque-forming units (PFU) or 0.2 ml of MEM containing 10 μg of poly(I:C) was dripped onto the tissue fragments. Control organ cultures received 0.2 ml of MEM. Adsorption of the inducers proceeded in the cultures for 1 hr while incubating at 35° in a 5% CO_2 environment. The cultures were then washed three times with 2 ml of medium 199 and replenished with 5 ml of medium 199 containing 0.3% bovine serum albumin and 50 μg/ml of gentamicin. Tracheal cultures were incubated for 16 hr at 35° in a humidified 5% CO_2 atmosphere. At the end of the incubation period, the culture fluid was collected by aspiration and stored at −20°. The tracheal fragments were also stored at −20°. Viability of the respiratory epithelium was observed by active ciliary movement. Control cultures retained this function through 7 days of incubation. Although cultures from mice exposed to ozone showed ciliary activity at the end of the incubation period for assay of interferon production, a decrease in the ciliary movement was noticed when compared with control cultures.

Interferon production in macrophage cultures. The monolayers of alveolar macrophages in culture dishes were inoculated with NDV at a multiplicity of infection of 10 or 1 ml of poly(I:C) containing 50 μg as interferon inducers. Control cultures received 1 ml of MEM. After adsorption for 1 hr, the cell cultures were washed three times with 2 ml of MEM and 5 ml of MEM containing 50 μg/ml of gentamicin was added. The cultures were incubated for 16 hr and the culture fluid was collected and stored as described for the tracheal cultures.

Interferon assay. Interferon from the organ cultures and the macrophage cultures was assayed by the plaque reduction technique using L-cells, with VSV as the challenge virus (9).

The interferon titer was expressed as the reciprocal of the highest dilution of the sample showing 50% plaque reduction. A standard reference mouse interferon (catalog number G002-90A-511) provided by National Institutes of Health was included in each assay. One unit of interferon in our assay was equivalent to two units of the reference interferon.

To assay for intracellular interferon from the organ culture the tracheal explants were three times frozen at −20° and thawed at room temperature. The explants were then ground in a Dounce homogenizer and the cellular components were suspended in 5 ml of MEM. The suspension was subjected to two more steps of freezing and thawing and then centrifuged at 15,000 rpm for 1 hr. The supernatant was collected and assayed for intracellular interferon.

The antiviral substance demonstrated in this study was considered to be mouse interferon for the following reasons. It was shown to be (i) stable at pH 2.0, (ii) nonsedimentable at 100,000*g* for 1 hr, (iii) inactivated by trypsin treatment, (iv) specific for host species, (v) inhibited by Actinomycin D, and (vi) inactivated at 56° for 1 hr (9, 10).

Results. The production of interferon from tracheal organ cultures of mice exposed to 0.8 ppm of ozone and mice exposed to ambient air is shown in Table 1. The data was obtained from two experiments. It was apparent that exposure of mice to 0.8 ppm of ozone for a period of 10 days did not affect the capacity of the tracheal epithelial cells to produce interferon *in vitro*. Both NDV and poly(I:C) served as

TABLE I. INTERFERON PRODUCTION BY TRACHEAL ORGAN CULTURES FROM MICE[a]

Days[b]	Interferon inducer	Interferon titer (units/4ml)			
		Ozone exposed mice (0.8 PPM)		Mice maintained in ambient air	
		Expt I	Expt II	Expt I	Expt II
0	None			0	0
	NDV			128	128
	poly(I:C)			64	64
4	None	0			
	NDV	64			
	poly(I:C)	64			
6	None		0		
	NDV		64		
	poly(I:C)		64		
8	None	0	0		
	NDV	32	64		
	poly(I:C)	64	64		
9	None		0		
	NDV		128		
	poly(I:C)		64		
10	None		0		
	NDV		128		
	poly(I:C)		64		
11	None		0		
	NDV		0[c]		
	poly(I:C)		0		
12	None	0	0		
	NDV	0	0		
	poly(I:C)	0	0		
14	None			0	0
	NDV			32	64
	poly(I:C)			32	128
15	None	0			
	NDV	0			
	poly(I:C)	0			
15		Remove to Ambient air			
17	None		0		
	NDV		0		
	poly(I:C)		0		
20	None		0		
	NDV		0		
	poly(I:C)		0		
21			Remove to ambient air		
24	None		0		
	NDV		0		
	poly(I:C)		0		
31	None		0		
	NDV		8		
	poly(I:C)		8		
32	None				0
	NDV				32
	poly(I:C)				64
39	None	0			
	NDV	64			
	poly(I:C)	128			

[a] Tracheal organ cultures from two mice were inoculated with either 10 μg of poly(I:C) or 5 × 10^6 PFU of Newcastle disease virus. After incubation at 35° for 16 hr culture fluids were adjusted to pH 2.0 with 2*M* perchloric acid and kept at 4° for 4 days. The resulting precipitate was removed by centrifugation at 15,000 rpm for 1 hr and pH of supernatant was readjusted to 7.0 with 2*M* NaOH. Interferon assay on culture fluids was carried out on L-cells using VSV as the challenge virus. Interferon titer is expressed as the highest dilution showing 50% plaque reduction.

[b] The day on which mice were sacrificed and tracheal organ cultures prepared.

[c] Probability that failure of interferon production was a result of chance less than 0.001. Calculated on the basis of the binomial distribution.

active interferon inducers. Also the tracheal cells were not releasing interferon at the time the animals were sacrificed since interferon was shown to be undetectable in the absence of an inducer. However, starting from the 11th day of exposure, there was an inhibition in the capacity of the organ cultures to produce interferon. This inhibition was detected in samples assayed on days 11, 12, 15, 17, and 20 from animals in the ozone environment. Assays performed on control samples and the macrophage samples during the period of inhibition indicated the specificity of the interferon depression for tracheal explants of ozone-exposed mice.

After the mice had been taken from the ozone chamber to ambient air, the impaired cellular function persisted for a time as shown in a sample taken after 3 days in the ambient air (Experiment II, Day 24). However, 10 days after the mice were removed to ambient air, the organ cultures had started to produce small amounts of interferon (Experiment II, Day 31). A sample studied after the animals had been in ambient air for 24 days revealed complete recovery to the normal level of interferon production (Experiment I, Day 39).

It is not possible to come to the conclusion that the ozone inhibited the production of interferon in organ cultures just by assaying the extracellular interferon. It is feasible that interferon might be produced but ozone inhibits its release, since it is known that certain chemicals such as *p*-hydroxymercuribenzoate affect the release of interferon without decreasing its production (11). Intracellular interferon was assayed to determine whether the absence of extracellular interferon could be due to an accumulation of the unreleased product by the cells. No interferon could be detected on the days on which extracellular interferon was not detected indicating that there was no accumulation of intracellular interferon.

Results obtained with alveolar macrophages were in marked contrast to those obtained from tracheal epithelium (Table II). The alveolar macrophages of mice exposed to ozone produced interferon as efficiently as the alveolar macrophages of mice kept in ambient air. Ozone exposure of mice for 21 days had no effect on the capacity of the alveolar macrophages to produce interferon. In the presence of interferon inducers the macrophages released interferon at equivalent titers without regard to their origin from ozone treated lungs or those exposed to ambient air.

Discussion. The ability of tracheal epithelium to respond normally to interferon inducers was lost after approximately 10 days in an elevated ozone environment. In one experiment (Experiment II) the unresponsive state persisted for 10 days while the animals were inhaling 0.8 ppm of ozone and for at least 3 days after returning to ambient

TABLE II. Interferon Production by Macrophage Cultures from Mice[a]

		Interferon titers (units/4ml)						Interferon titer (units/4ml)			
		Ozone exposed mice (0.8 PPM)		Mice maintained in ambient air				Ozone exposed mice (0.8 PPM)		Mice maintained in ambient air	
Days[b]	Interferon inducer	Expt I	Expt II	Expt I	Expt II	Days	Interferon inducer	Expt I	Expt II	Expt I	Expt II
0	None			0	0	21	None		0		
	NDV			512	256		NDV		256		
	poly(I:C)			512	256		poly(I:C)		512		
4	None	0				21			Remove to ambient air		
	NDV	512									
	poly(I:C)	256									
6	None		0			24	None		0		
	NDV		256				NDV		256		
	poly(I:C)		256				poly(I:C)		256		
8	None	0	0			25	None	0			
	NDV	512	256				NDV	512			
	poly(I:C)	256	256				poly(I:C)	256			
12	None	0	0			31	None		0		
	NDV	512	256				NDV		256		
	poly(I:C)	256	256				poly(I:C)		256		
14	None			0	0	32	None	0			0
	NDV			1024	256		NDV	512			512
	poly(I:C)			256	256		poly(I:C)	256			256
15	None	0				33	None			0	
	NDV	1024					NDV			512	
	poly(I:C)	512					poly(I:C)			512	
15		Remove to ambient air				39	None	0			
							NDV	512			
							poly(I:C)	512			
17	None	0	0								
	NDV	512	256								
	poly(I:C)	512	256								

[a] Macrophage cultures were prepared from lung lavages of 18 mice. Macrophage cultures were inoculated with either 50 μg of poly(I:C) or Newcastle disease virus at a multiplicity of 10. Culture fluids were collected and processed for interferon titration as described for the tracheal organ cultures.

[b] The day on which mice were sacrificed and macrophage cultures prepared.

air. When tested after 10 days in ambient air a partial ability to respond had returned. Experiment I indicated a complete return to normal interferon production upon induction in a test performed 24 days after return of the mice to ambient air. The impaired cellular function was shown for tracheal epithelium, but it is likely that the effect extended well into more distal airways.

The mechanism by which ozone inhibited the production of interferon by tracheal cells *in vitro* remains to be determined. It is possible that ozone inhibited interferon production at the level of transcription or translation. It has been reported by Werthamer *et al.* (2) that exposure of mice to 2.5 ppm daily for 2 hr resulted in a decrease of lung RNA synthesis with an increase in lung protein synthesis during the first 20 days of exposure. The increase in protein synthesis was thought to be associated with a replacement of lost structural (membrane) protein. Those events might in turn affect the formation of other proteins such as interferon. It is known that the synthesis of interferon messenger RNA and its subsequent translation is necessary for interferon production (12). Thus, any decrease in the synthesis of RNA may also affect the production of interferon. In the current experiment the epithelial cells were capable of normal interferon production upon induction for approximately 10 days in the high ozone environment before becoming unresponsive. The finding suggests exhaustion of a needed synthetic step or possibly an alteration in membrane structure on replacement epithelium in response to the oxidizing effect of ozone.

Macrophages washed from the lungs and manipulated *in vitro* for approximately 3 hr prior to contract with interferon inducers were found to be uniformly responsive. There was no significant difference in the titers of interferon from macrophage cultures of mice kept in ambient air and mice exposed to ozone. Valand *et al.* (13) reported that exposure of rabbits to 25 ppm NO_2 for 3 hr inhibited the capacity of the alveolar macrophages to produce interferon. They concluded that the inability of the NO_2 exposed macrophages to produce interferon was not due to a defect in adsorption, penetration, or uncoating of the virus, or to a loss of RNA synthesis (14). It is difficult to compare our results with those of Valand *et al.* (13, 14) as the inducer, method of interferon assay, and oxidant pollutant employed were all different.

Interferon has always been considered to play an important role in viral infection. During many viral infections interferon is present prior to antibody formation, and it has been suggested that interferon is the first host defense process to be mobilized. A number of viral vaccines and synthetic interferon inducers have been reported to have the potential of inducing interferon production. Panusaran *et al.* (15) demonstrated that instillation of an interferon inducer, CP20,961 *N,N*-Dioctadecyl-*N′,N*-bis-(2 hydroxyethyl) propanediamine, in the form of nose drops prevented the symptoms of rhinovirus infection and virus shedding in volunteers. The procedure was associated with the production of elevated interferon titers in the nasal washing. Administration of aerosolized poly(I:C) in mice was found to be effective against influenza infections (16). A number of viral vaccines have been investigated for potential use as interferon inducers in man (17, 18). In an experiment where volunteers received influenza A/Bethesda/68 (H_3N_2) wild-type virus, Murphy *et al.* (17) reported that six of seven volunteers had significant levels of interferon in their nasopharyngeal washings. In view of our findings, the environmental factors such as the level of atmospheric ozone may have to be taken into consideration if interferon inducers are to be used widely in the treatment and prophylaxis of viral infections.

Summary. The effects of 0.8 ppm ozone on the capacity of the tracheal epithelium and alveolar macrophages of mice to produce interferon *in vitro* was studied. Exposure of mice to ozone for a period of 11 days or more affected the capacity of the tracheal epithelial cells *in vitro* to produce interferon. The inability of the tracheal epithelium *in vitro* to produce interferon was not due to the inhibition in the release of intracellular interferon but to an inhibition in the production of interferon. There was a complete recovery of the ability of tracheal epithelium to respond to interferon inducers

after the mice were returned to ambient air 24 days post ozone exposure. However, ozone did not seem to have any affect on the capacity of the alveolar macrophages to produce interferon *in vitro*.

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in-Stimulated Spleen Cells: Dissociation between DNA Synthesis and IgM Production at the Cellular Level[1] (39424)

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xin (LPS) induces DNA synthesis .is followed by IgM production in murine B-lymphocytes (1–9). it appears that DNA synthesis is 'equisite for IgM production. An- ıd Melchers reported that hydrox- u)-treated spleen cells responded ith increased IgM synthesis while dine incorporation was reduced n 99% (10). This observation supports a dissociation of DNA and IgM production, but to our e a direct demonstration of this at ar level has not been reported. ıethod which permitted the simul- .etection of cytoplasmic IgM and dine incorporation in individual eport here that inhibition of DNA by Hu does not result in an appre- luction in the percentage of cells ng IgM. In addition, the cells mak- ıave not incorporated [^{3}H]thymi-

ls and methods. Animals. C57Bl/ : mice were obtained from Jackson Harbor, Maine.

t and endotoxin. LPS was ex- ıd purified from *S. typhimurium,* STM-7) by the phenol–water pro- 1).

tion of spleen cells. Mouse spleen prepared and cultured by the pro- Alder *et al.* (12). Hydroxyurea, 'hen used, was added at the begin- ultivation. Control cultures con-) spleen cells plus Hu, (ii) spleen cells plus LPS, and (iii) spleen cells alone. One microcurie of [^{3}H]thymidine (Schwarz-Mann, Orangeburg, N.Y., 14.8 Ci/mmole) was added initially to each culture (2×10^6 spleen cells in 1 ml of RPMI-1640 containing 5% heat-inactivated normal human serum and antibiotics).

Fluorescent-autoradiographic procedure. Cells were removed from culture after 48 hr of LPS-stimulation and washed twice with RPMI-1640. Washed cells were deposited on microscope slides in a cytocentrifuge (Shandon Scientific Co., Sewickly, Pa.), fixed in ethanol for 1 min, and air-dried. Fluorescent antimouse IgM (fluorescein-conjugated heavy chain-specific, Meloy Laboratories, Springfield, Va.) was then layered over the cell pellet, and incubated at room temperature for 30 min. After washing with phosphate-buffered saline, preparations were either viewed directly on a fluorescence microscope or used in the combined fluorescent-autoradiography procedure (13). Best results were obtained by alternate dark field and fluorescence microscopy, utilizing a Leitz Ortholux microscope equipped with an Osram HBO 200 W light source, BG 38 and BG 12 excitors and a 470 or a 490 barrier filter.

[^{3}H]thymidine incorporation. Triplicate determinations of [^{3}H]thymidine incorporation were made after 48 hr of LPS-stimulation by TCA precipitation of washed cells, followed by solubilization and scintillation counting (12).

Autoradiography. Cytocentrifuged cells were treated with fluorescent anti-IgM and were dipped into Kodak NTB-3 emulsion (13). After optimal exposure, slides were developed, fixed, washed, dried and cover-slipped using phosphate-buffered saline-glycerine, 1:9. Cells were scored for fluorescence (cytoplasmic IgM) and [^{3}H]thymidine incorporation (grains over nuclei). This pro-

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ed by a postdoctoral fellowship from and French Laboratories, Philadelphia, ıddress: VA Hospital, 151, Research, 54th Ave. South, Minneapolis, Minn. 55417.

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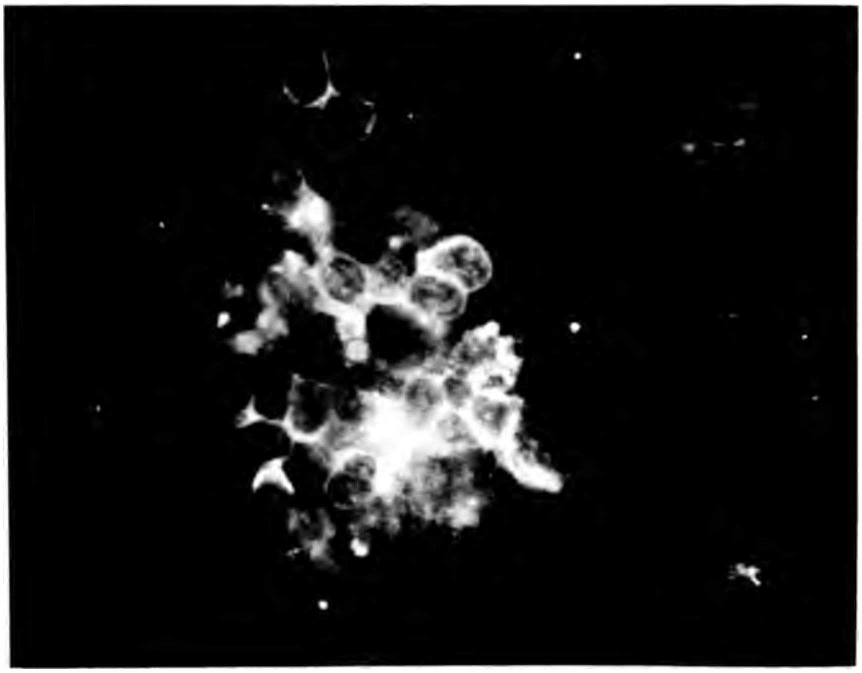
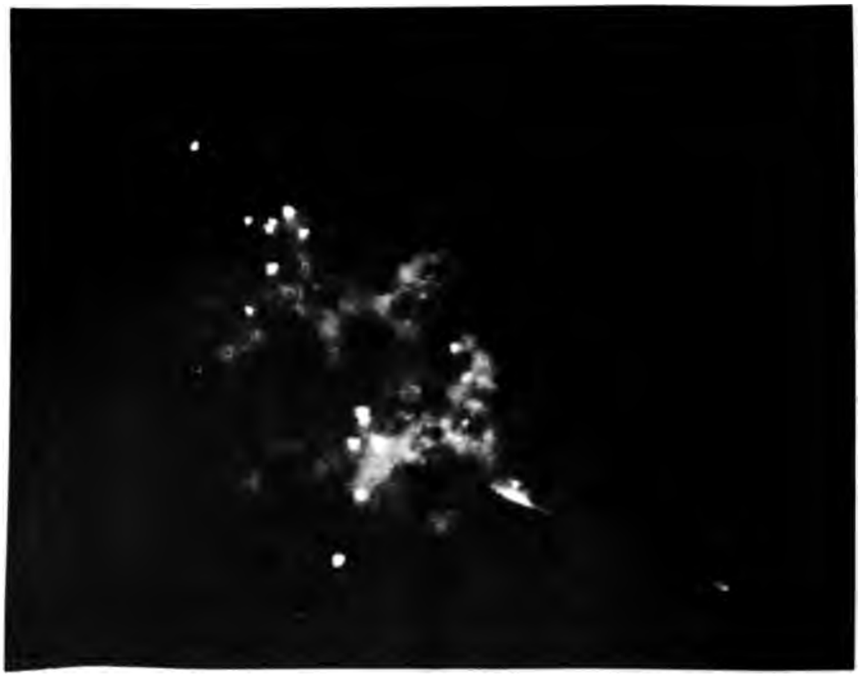




FIGS. 1 and 2. Combined fluorescent autoradiography of LPS-stimulated spleen cells cultured with Hu; Fig. 1, focused on cells, Fig. 2, focused at the plane of the emulsion. ×648.

.ure permits the direct assessment of IgM
ıtent and [^{3}H]thymidine uptake by indi-
ual cells.

lesults. The results in Table I show that
continuous presence of Hu in 48 hr LPS-
nulated cultures inhibited [^{3}H]thymidine
orporation more than 98%. Autoradiog-
hy showed that Hu blocked the uptake of
[]thymidine by cells which stained posi-
:ly for cytoplasmic IgM (Figs. 1 and 2).
the other hand, spleen cells stimulated
LPS to produce IgM in the absence of Hu
orporated [^{3}H]thymidine (Fig. 3) in the
viously reported fashion (13). It is clear,
refore, that at a cellular level LPS can
uce IgM production by murine B cells in
absence of preceding DNA synthesis.

ABLE I. [^{3}H]THYMIDINE INCORPORATION BY LPS-
MULATED CELLS IN THE PRESENCE OR ABSENCE OF
HYDROXYUREA[a]

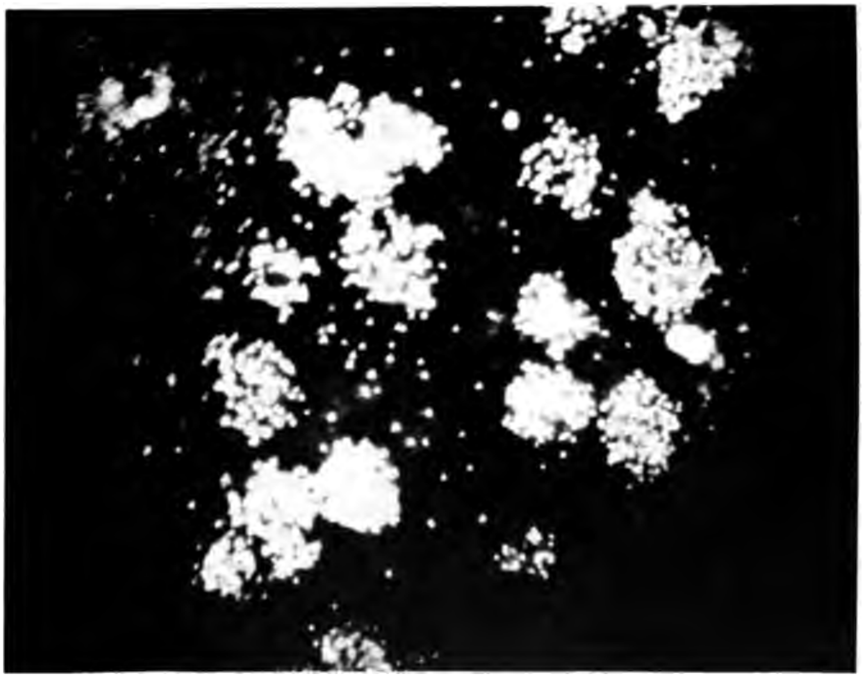
	CPM × $10^{-3}/2 \times 10^6$ cells ± SD		
.	Control	10^{-2} *M* Hu	Percent reduction
	143 ± 4.9	1.64 ± .32	98.9
	242 ± 24.7	4.13 ± .51	98.3

LPS added to conc of 10 μg/ml.

These observations confirm the report of Andersson and Melchers (10).

Surprisingly, the fluorescent antibody procedure showed that approximately 50% of the 48 hr stimulated spleen cells contained IgM whether or not Hu was present in the cultures. One would expect proliferation of the B cells to increase the percentage of IgM producers in cultures not inhibited by Hu. The observation is inexplicable at present. However, there are several possibilities, including dissimilar maturation to IgM production and restriction of cell proliferation by [^{3}H]thymidine of high specific activity.

Summary. LPS stimulation of mouse spleen cells in the presence of Hu resulted in almost total suppression of [^{3}H]thymidine incorporation without affecting the percentage of cells induced to produce IgM. Utilizing a method which permitted the simultaneous measurement of IgM production and [^{3}H]thymidine incorporation in individual cells, it was demonstrated directly that LPS stimulation of IgM production can occur in the absence of DNA synthesis.



ɔ. 3. Same procedure used on LPS-stimulated spleen cells cultured without Hu. The photograph is focused
: plane of the emulsion. ×648.

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Kinetics of Immunoglobulin Transport into Canine Bronchial Secretions (39425)

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The established use of periodic gamma globulin injections to help control sino-pulmonary infections in patients with hypo- or agammaglobulinemia (1-3) suggests that antibody from the intravascular space gains access to lung tissue and respiratory secretions. These commercial preparations of gamma globulin contain primarily immunoglobulin G (4). It has been established that IgG is a major constituent of the protein milieu of the lower respiratory tract in humans (5-7) and a variety of animals including the rabbit (8), dog (9-11), and rat (12). Other immunoglobulins such as IgA and IgE are present, but IgM is rarely found (7) or, if so, in very small amounts (11). *In vitro* studies of bacterial phagocytosis by alveolar macrophages obtained from humans (13) and rabbits (14) have shown opsonic IgG antibody to enhance bacterial uptake by these cells and thus help explain the apparent usefulness of gamma globulin therapy (3).

This present study examines the transfer of intravascular IgG into broncho-alveolar secretions, the kinetics of this protein in the lower respiratory tract, and the effect of altering the IgG molecule on this transfer. Normal dogs have been used.

Materials and methods. Animals. One-year-old male beagle dogs weighing approximately 10-15 kg were used. Animals were housed individually in cages which were designed to collect urine specimens.

Experimental design. To study the plasma to broncho-alveolar fluid transfer of parenterally administered immunoglobulins, groups of dogs were injected with ^{125}I-labeled purified proteins and the distributions of radioactivity evaluated. After intramuscular injection, serum samples were taken at 2-hr intervals for the first 8 hr, then daily (9 AM) for the first week and twice weekly thereafter. The urine was monitored daily for ^{125}I activity. Pulmonary lavage was performed 8 hr after injection of labeled protein, daily for the first week, and twice weekly thereafter.

Preparation of immunoglobulin G and M for injection. Methods used to obtain canine serum IgG and IgM have been described (15, 16). In brief, pooled dog serum was precipitated with 30% dry ammonium sulfate; the precipitate was redissolved and dialyzed extensively against borate-saline buffer to remove excess ammonium sulfate. After a final dialysis in 0.02 *M* tris(2-aminohydroxymethyl-1,3-propanediol) HCl buffer, the preparation was chromatographed on DEAE-cellulose and eluted with a 0.3 *M* NaCl-Tris gradient. IgG containing fractions of low molarity (conductivity 1.0-9.0 mmho) and pH 8.0-8.5 were collected and gel filtered through Sephadex G-200. Effluent protein was identified as IgG by the criteria of cathodal electrophoretic mobility on immunoelectrophoresis, immunoprecipitation with specific anti-Fc gamma (γ) chain antiserum (15), and a sedimentation coefficient of 6.7S found with ultracentrifugation (16). IgG was principally of the subclass γa,b (15).

IgM was isolated from the same serum preparations except that protein fractions of conductivity 12-16 mmho and pH 9.0 after DEAE chromatography were pooled. This material was passed over a Sepharose 2B rabbit anticanine IgM immunoadsorbent (17) and IgM was eluted with 0.1 *M* citrate, pH 2.2. Finally, IgM was gel filtered through Sepharose 6B and concentrated with positive pressure ultrafiltration. Immunoglobulin preparations were stored at 4° until iodinated.

F(ab')$_2$ fragments of IgG were made by pepsin digestion (14). Purified IgG was digested with pepsin (Worthington Biochemical Co., Freehold, NJ) at pH 4.3 for 8 hr

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and gel filtered on Sephadex G-150. Sedimentation coefficients for the fragments were determined by ultracentrifugation (Beckman model E).

Protein iodination. Purified serum immunoglobulins were labeled with carrier-free ^{125}I (New England Nuclear, Boston, Mass.) by the method of McConahey and Dixon (18) using choloramine-T and sodium metabisulfate. The excess ^{125}I was removed by extensive dialysis against 0.9% saline buffer. The specific activity of the preparations used ranged from 7.85×10^7 to 1.73×10^8 cpm/mg. Each animal received a 3-ml intramuscular injection containing 2.5 mg of radiolabeled purified immunoglobulin.

Collection and processing of specimens. Selective lavage of lower lung lobe was done through a fiberoptic bronchoscope as described previously (19). The bronchoscope was wedged in a lower lobe bronchus and two 50-ml aliquots of sterile 0.9% saline were infused and aspirated into a sterile syringe. Sequential washes on successive days were done in alternate lower lobes. All animals tolerated the procedure well and had no noticeable adverse effects.

The lavage fluid was centrifuged at 500*g* for 15 min at 25° and the supernatant was decanted from the cell pellet. The percentage of red blood cells in the pellet was consistently less than 3% in analyzed specimens. A 1-ml aliquot of the lavage fluid was analyzed for radioactivity in an automatic gamma counter (Series 1185, Nuclear Chicago Corp., Des Plaines, IL). In certain experiments the entire supernatant fluid was concentrated to a 2–3 ml vol with positive pressure ultrafiltration (UM-10 filter, Amicon Corp., Lexington, MS) at 4°.

Serum was extracted from clotted blood. Twenty-four-hour urine samples were collected from animal cages, and after the volume was measured, 1 ml was examined for radioactivity.

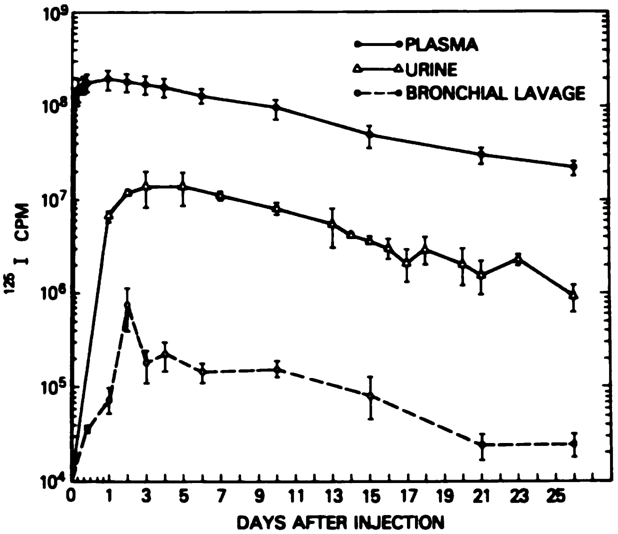
Sucrose density ultracentrifugation (8, 16) was used to separate serum and bronchial proteins. Samples (0.25 ml) were layered on 5-ml linear sucrose-density gradients (10–40%) and centrifuged at 35,000 rpm for 18 hr at 4°. Fractions of 0.2 ml were collected and counted in a gamma counter to identify the position of the labeled protein in the serum and bronchial secretions. Immunoglobulins were identified in gradient fractions by gel diffusion, using antisera specific for each protein. Rabbit anti-IgG Fc and Fab antisera were used (15). It was felt that ultracentrifugation provided a more accurate identification of labeled immunoglobulins than nonspecific precipitation of protein.

Calculations. Data were plotted semilogarithmically and the slopes of curves determined by the method of least squares. Student's *t*-test was used to compare the decay curve slopes. The half life was derived from the equation for exponential decay (20), with corrections made for ^{125}I decay during the time of observation. The plasma volume was assumed to be 7% of each animal's body weight (21). The urine activity was corrected to total urine volume on the basis of 24 hr urine collection.

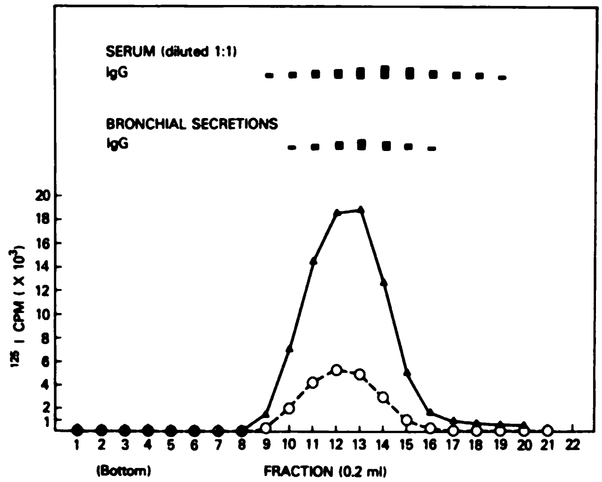
Radioactivity in broncho–alveolar washings was corrected to total bronchial surface area. It was determined in a group of control animals that the 100 ml lower lobe lavage consistently sampled about 25% of the bronchial surface area. When an equivalent amount of radiopaque dye was infused and aspirated through the bronchoscope, chest X-rays taken immediately after removal of the dye showed selective opacification of the lower lobe area. Autopsy and *in vitro* lung infusions were used to verify this estimate.

Results. Reproducibility of broncho-alveolar lavage. Seventy-five 100-ml lavages were performed in these studies, and the mean recovery of fluid was 78 ± 0.77 ml (range, 61–90 ml). To document the consistency of protein values obtained by serial bronchial lavage, two dogs were lavaged daily for 5 successive days and the albumin and IgG values in concentrated lavage fluid specimens (mean final volumes 2.8 ml ± 0.2 SEM) measured. There was a variation of 5–8% in the daily values of these two proteins with repeated lavage. The mean ratio of IgG (mg/ml) to albumin (mg/ml) was 0.06 ± 0.003 for the 10 lavages and was similar in both animals. Serum values for IgG and albumin showed no fluctuation during the 5 days of lavage.

Distribution of radiolabeled immunoglobulin. The pattern of distribution of IgG is n Fig. 1. Each point represents the tic mean and standard error of detions from four dogs. Rapid absorp- intramuscularly administered IgG ed by 2–4 hr with maximal plasma evident at 24 hr, followed by gradinution over the next 25 days. At 8 ır there was evidence of some radio- in the bronchial secretions but maximal activity was seen at 48 hr, followed by an elimination rate during the next 3 weeks that was not significantly different ($p > 0.20$) from that in serum. Figure 2 shows a representative separation in sucrose density gradients of serum and concentrated bronchial secretions obtained 48 hr after injection of labeled IgG. Greater than 95% of the radioactivity is associated with gradient



Distribution of radioactivity after intramuscular administration of ^{125}I-labeled IgG in plasma, urine, hial lavage fluid is shown for 26 days of observation. ^{125}I activity depicted on the ordinate has been to total plasma volume, urine volume, and bronchial surface area. Each point represents the mean and rror of the activity found in four dogs.



Separation is shown for serum and concentrated bronchial secretions obtained 48 hr after intramuscu- ɔn of radiolabeled IgG on identical 10 to 40% linear sucrose density gradients. Vertical bars give relative ntrations in the gradient fractions. ^{125}I activity measured on the ordinate coincides with the sedimenting f IgG in both the serum (▲– –▲) and bronchial secretion (O– –O) gradients.

fractions containing IgG in both specimens, confirming that nonspecific radioactivity or protein degradation products are not responsible for the measured ^{125}I activity. However, in urine samples radioactivity was nonprecipitable and not attached to identified protein moieties.

The elimination of radiolabeled IgG from the serum of this group of dogs was 9 days ($T_{1/2}$, Table I). In the lavage specimens, the $T_{1/2}$ of 7.5 days was not statistically different from serum ($p > 0.20$). Cumulative results from the 26 days of study indicated that 0.15% of the initial IgG dose was recovered in the lung secretions.

Pepsin-digestion of IgG produced $F(ab')_2$ fragments with a sedimentation coefficient of 5.5 $s_{20,w}$. After labeling with ^{125}I, these fragments were injected into a pair of dogs and the distribution monitored (Fig. 3). Again, there is rapid absorption of the intramuscularly administered protein and maximal radioactivity in bronchial secretions is seen 24 hr after injection, although some activity is evident at 8 hr. Most of the radioactivity in the bronchial secretions was gone by 10 days after injection of labeled protein. Repeated sucrose density gradient separations of serum and concentrated bronchial secretions from these dogs indentified radioactivity in the $F(ab')_2$ fractions of the gradients. Cumulatively, only 0.03% of initial dose of $F(ab')_2$ fragments were recovered in lung lavage specimens. As given in Table I, the elimination rates in serum and lavage were much less for the fragments than intact IgG.

Similar studies in a pair of dogs were done with IgM. The kinetics of IgM absorption were similar to those found for IgG except the elimination rate from serum was less. No IgM associated radioactivity was detected in broncho-alveolar lavage specimens.

TABLE I. HALF-LIFE OF ^{125}I-LABELED PROTEINS IN SERUM AND LUNG LAVAGE.

	Serum	Broncho-alveolar lavage
IgG ($n = 4$)	9.0 ± 0.08[a] (8.8-9.2)	7.5 ± 0.38 (6.5-8.3)
F(ab') ($n = 2)^2$	4.3[b]	1.8
IgM ($n = 2$)	5.2	

[a] Mean $T_{1/2}$ in days corrected for ^{125}I decay ± SEM.

[b] Average value.

Discussion. The objective of this study was to determine the kinetics of immunoglobulins entering the lower respiratory tract secretions from the intravascular space in normal animals. In this respect a very small percentage (0.15%) of parenterally administered IgG was recovered in broncho-alveolar lavage specimens during approximately 4 weeks of observation. Alteration of the IgG molecule to create an $F(ab')_2$ fragment, devoid of the Fc portion of the gamma chain, effectively reduced the transudation of IgG into respiratory secretions to an almost negligible amount. Moreover, intact IgM did not appear in the broncho-alveolar lavage.

We conclude from these studies in dogs that circulating humoral IgG contributes only a small portion of the immunoglobulin found in pulmonary secretions. Thus, it appears that passive infusion of IgG is not an efficient method of increasing IgG in respiratory secretions of normal subjects. However, the critical level of specific antibody in respiratory secretions needed for protection against infectious agents is now known and may be quite small. Furthermore, pulmonary irritation or suppuration undoubtedly cause changes in pulmonary vascular permeability and serve to increase concentrations of immunoglobulins in respiratory secretions.

It was of interest that peptic digestion of IgG to create a smaller protein fragment (5.5S) devoid of the Fc portion reduced transudation or penetration into lung secretions. The Fc portion of the molecule may be critical for determining the extravascular distribution of IgG as well as other biological activity of the immunoglobulin. In rabbits (14), $F(ab')_2$ fragments generated from IgG having specific antibody activity for *Pseudomonas aeruginosa*, retain agglutinating activity and opsonizing potential. However, these antibody fragments are ineffective in promoting phagocytosis by alveolar macrophages because the antibody cannot attach to the Fc membrane receptor of the macrophage and initiate bacterial ingestion.

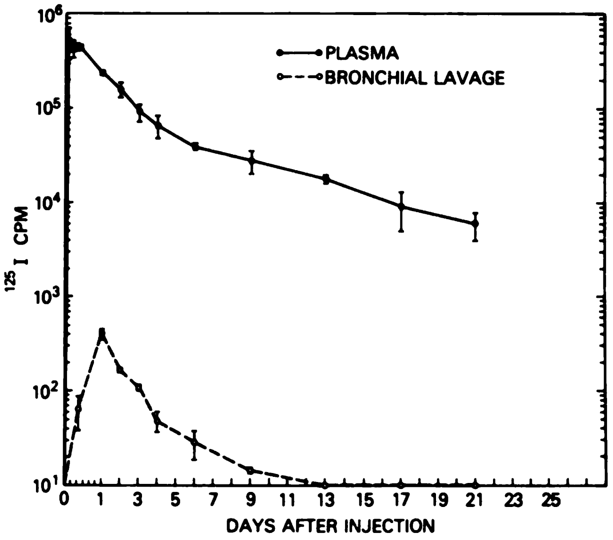


FIG. 3. Distribution of radioactivity after intramuscular administration of ^{125}I-labeled F(ab')$_2$ fragments of IgG in serum and bronchial lavage fluid is shown for 21 days of observation. ^{125}I activity on the ordinate has been corrected to total plasma volume and bronchial surface area. Each point represents an average value and the range found.

The intravascular clearance of canine IgG was similar to that previously reported for gamma globulin. The calculated $T^1/2$ of 9.0 days for IgG in this study is comparable to that of 7 (22) and 8 (23) days found in earlier studies using dog gamma globulin. The rate of clearance of dog IgM and F(ab')$_2$ fragments have not been studied previously. Studies with mouse (24) and human (25, 26) IgG have shown that the catabolic rate of these proteins is directly proportional to the concentration of IgG in plasma, and that the Fc portion of the molecule appears to be the structural configuration critical for this concentration dependent catabolism. In contrast, this requirement does not occur in the guinea pig (27). Such factors affecting catabolism of dog IgG are not known at present. The rapid catabolic rate for human F(ab')$_2$ fragments of 5 hr (28) was not duplicated in our study, but the $T^1/2$ value of 4.3 days (Table I) is appreciably shorter than for intact dog IgG.

Summary. Radiolabeled ^{125}I preparations of canine IgG, IgM, and F(ab')$_2$ fragments of IgG were injected intramuscularly into normal dogs to quantitate the transfer of immunoglobulins from the intravascular space into the lower respiratory tract and to observe the clearance of these proteins from lung secretions. Respiratory fluids were sampled by serial broncho-alveolar lavages during a 3-4 week interval. Only a small portion (0.15%) of the passively administered IgG was recovered in respiratory specimens indicating that minimal transfer of humoral IgG occurs under normal conditions.

Alteration of the IgG molecule by removal of the Fc portion almost eliminates its penetration into lung secretions. Furthermore, IgM, which is not a prominent component of normal lung secretions, is not detected in these secretions following passive intramuscular injection.

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enic strains of Mice Susceptible and Resistant to Mouse Hepatitis Virus[1]

(39426)

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ince Snell (1) established congenic f mice which differ on the basis of or histocompatibility antigen, the ss of such lines for both genetic and gical studies has grown in impor- -4). We here report the establish- the line of C_3HSS mice which are le to a standard strain of mouse virus, derived from PRI mice RI) and which are congenic to the ed inbred line of resistant C_3H ese strains of mice should be useful in analyzing the locus for suscepti- t also should allow for lymphocyte rophage transfer so that the factors in pathogenesis may be more accu- alyzed.

lent variant of the original virus RI) which is capable of killing both le and resistant strains of mice was by Shif and Bang (5). This complex endence of virulence and suscepti- epresented diagramatically in quad- lysis (Fig. 1), a presentation which r to that used by plant pathologists his there is a matching of one gene e in host susceptibility against the d one-step conversion of virus from n to PRI mice to adaption to C_3H e present paper is limited to a study tibility to MHV-PRI.

als and methods. Mouse strains. n (PRI) and C_3H mice were used. e were originally obtained from Dr. lson in 1951 and have since been ed by brother-sister inbreeding in ratory. This strain of mice is geneti- sceptible to mouse hepatitis virus . C_3H, a resistant strain, was ob- om Dr. H.B. Andervont on August 6 and has also been maintained inbreeding.

Virus. MHV-2 strain of mouse hepatitis virus was obtained from Dr. John Nelson (7). It was passed in our laboratory by intraperitoneal inoculation of one-month old PRI mice. A 10% (wt/vol) liver homogenate was prepared by grinding up livers of moribund mice. This preparation was used as stock virus and kept at −70°.

Macrophage cultures. Cultures of peritoneal macrophages were prepared in the following manner: Mice were stimulated by injecting 3.0 ml of sterile thioglycollate medium (Difco) intraperitoneally. Three days later, 5.0 ml of warmed phosphate buffered solution (pH = 7.2) containing 20 U.S.P. units of sodium heparin (Upjohn) per milliliter was again injected intraperitoneally. Fluid was withdrawn and cells were counted. The cell suspension was centrifuged at 1000 rpm for 10 min and the cellular pellet was then resuspended in Chang's medium which is 90% horse serum, 8% Hanks BSS, and 2% beef embryo extract. The medium was seeded with $1\text{-}1.5 \times 10^6$ cells in Wassermann tubes which were then closed with siliconized stoppers. After attachment, they were transferred to a roller drum in a 37° incubator.

Tests of histocompatibility. Histocompatibility was tested by reciprocal skin grafting and mixed lymphocyte culture (MLC) reaction.

Method for obtaining a congenic susceptible line of mice. Inbred PRI mice were crossed with inbred C_3H mice to produce F_1 hybrids. The hybrids were then mated to the resistant strain (C_3H) to produce back-cross generation-1 (BC-1). The progeny of this back-cross were tested for cell susceptibility, and only the susceptible ones were chosen and mated again to C_3H mice to produce BC-2. This was continued until BC-20 was achieved. During each generation, susceptibility to MHV was tested by inoculating cultured macrophages with MHV at a dilu-

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ed.

Host / Virus	C_3H	C_3H_{SS}
MHV-PRI	−	+
MHV-C_3H	+	+

FIG. 1. Quadrate analysis. Plus signs indicate that the virus and the host are compatible, whereas the negative sign indicates that they are not compatible. C_3HSS = C_3H mice carrying the gene for susceptibility to mouse hepatitis virus (MHV). MHV-PRI = original strain of MHV, derived from the livers of moribund PRI mice; MHV-C_3H = virulent viriant virus.

tion of 10^{-3}, since Bang and Warwick (8) had found that cultured peritoneal macrophages expressed the genetic response to MHV of their donor animals. Mice with susceptible macrophages were kept for further breeding.

Method for obtaining an homogenous C_3H-susceptible (C_3HSS) line of mice. Susceptible mice from BC-20 were bred by brother-sister mating: BC-20 × BC-20 = Ss × Ss → SS,2Ss,ss. ss was eliminated by testing cultivated peritoneal macrophages and discarding all mice that were resistant. Homozygosity for susceptibility was tested by back-crossing mice with susceptible macrophages to resistant C_3H mice. Mice whose descendants were 100% susceptible were kept as parents for subsequent inbreedings.

SS × C_3H = SS × ss → Ss ——100% susceptible
Ss × C_3H = Ss × ss → Ss,ss —— 50% susceptible

By selection of presumed homozygotes, C_3HSS was developed.

Results and Discussion. The ratio of susceptible to resistant mice among 20 back-crosses. The ratio of susceptible to resistant mice among the offspring of back-crosses was rather constant, close to 1:1 (Table I). This suggests that the genes segregated in a typical Mendelian fashion with one locus responsible for susceptibility. However, the overall percentage of mice yielding positive cultures was 209/455 or 45.9. If the first back-cross which still contains a considerable mixture of genes is eliminated, there was no significant difference in the expected and the actual ratios of susceptible to resistant mice.

TABLE I. RESULTS OF *IN VITRO* INOCULATION OF MOUSE MACROPHAGE CULTURES FROM SUCCESSIVE BACK-CROSS MATING OF SUSCEPTIBLE MICE TO A RESISTANT STRAIN.

Generation	Susceptible/ total mice	Susceptible (%)	Expected susceptible (%) on hypothesis of one dominant gene
F_1 (PRI × C_3H)	21/21	100	100
Back-cross-1	6/27	22.2	50
Back-cross-2	2/5	40.0	50
Back-cross-3	11/18	61.1	50
Back-cross-4	3/7	42.9	50
Back-cross-5	3/10	30.0	50
Subtotal	25/67	37.3	
Back-cross-6	15/32	46.9	50
Back-cross-7	14/30	46.7	50
Back-cross-8	25/43	58.1	50
Back-cross-9	7/15	46.7	50
Back-cross-10	9/17	52.9	50
Subtotal	70/137	51.1	
Back-cross-11	3/6	50.0	50
Back-cross-12	18/32	56.3	50
Back-cross-13	16/36	44.4	50
Back-cross-14	3/7	42.9	50
Back-cross-15	15/31	48.4	50
Subtotal	55/112	49.1	
Back-cross-16	6/13	46.2	50
Back-cross-17	16/38	42.1	50
Back-cross-18	11/23	47.8	50
Back-cross-19	6/17	35.3	50
Back-cross-20	20/48	41.7	50
Subtotal	59/139	42.4	
Grand total	209/455	45.93	

Test for histocompatibility. (i) *Skin graft.* After 10 generations of C_3HSS and C_3H, 20 reciprocal skin grafts were exchanged between C_3H and C_3H_{SS}. Ten mice were kept for 45 days while others were kept for 75 days. There was no rejection between them. Exchanges between PRI and C_3H, PRI and C_3HSS were rejected within 9 to 12 days. (ii) *Mixed lymphocyte culture reaction.* The MLC reaction was kindly performed by the Immunogenetics Laboratory of The Johns kins University School of Medicine. e was no stimulation between C_3H and SS mice.

rther testing of susceptibility of C_3HSS to MHV. Even though C_3HSS was de- ped after four generations of inbreeding presumed homozygotes and no hetero- tes were found thereafter (Table II), a ine check-up of our inbred C_3HSS mice nies has been done to ensure the homo- city. Forty-five mice, 4 to 6 weeks of were given 0.2 ml of 4.5 log ($TCID_{50}$) rus intraperitoneally. They all died in 4 . Macrophage cultures prepared from dividual mice were also tested by inoc- ng 10^{-5} to 10^{-7} dilutions of virus and found susceptible. When a complete assay was performed, the viral titer the same as from PRI mice.

ange of susceptibility in C_3HSS mice. ily *et al.* (9) demonstrated that the abil- o resist the lethal dose of MHV-PRI tion of C_3H mice was a characteristic of dult. That is, MHV-PRI infection was l to infant C_3H mice; their macrophages orted viral growth and were destroyed e virus. Since C_3HSS and C_3H are con- , it was of interest to determine her there was any change in susceptibil- f C_3HSS with increasing age. Differ- ges of mice were tested by infection of ophage cultures and/or by intraperito- injection of MHV-PRI. There was a ease in susceptibility as the mice passed eks of age (Table III). However, mice een 12 and 13 months old apparently most of this reduced susceptibility and me susceptible again. But when 44 ophage cultures from various ages of were tested, they were found uni- ly susceptible.

mmary. A congenic strain of C_3HSS , which is histocompatible with C_3H mice but differs from them in susceptibility to mouse hepatitis virus (MHV), has been developed by introducing the gene for susceptibility to the MHV-PRI virus from the PRI mice. This was accomplished by continual back-crossing of the hybrids to the C_3H mice, but at the same time by selection of susceptibility by use of macrophage culture tests. After 20 back-crosses, a strain homozygous for susceptibility was produced by brother–sister mating of individual mice whose potential for carrying the recessive gene for resistance was tested in progeny. Since the original choice of mice for breeding was based on *in vitro* macrophage susceptibility, and since highly susceptible mice were developed on the same basis, it seems evident that macrophage susceptibility is an integral aspect of mouse susceptibility.

TABLE II. RESULTS OF PROGENY TEST FROM ESUMED HOMOZYGOTE SUSCEPTIBLE C_3H MICE.

erations[a]	Susceptible/total mice	Susceptible (%)
F_1	50/81	61.7
F_2	70/90	77.8
F_3	324/358	90.5
F_4	76/77	98.7

and F_2 were tested *in vitro*; F_3 and F_4 were tested o.

TABLE III. RESULTS OF *IN VIVO* INOCULATION OF VARIOUS AGE C_3HSS WITH MHV-PRI.[a]

Age of mice	Number tested	Number died	Number died/number tested (%)
4 weeks	10	10	100
5 weeks	10	10	100
6 weeks	27	27	100
7 weeks	18	18	100
8 weeks	22	9	41
9 weeks	10	1	10
12-13 months	34	32	94

[a] Macrophages from survived mice were tested individually and found susceptible.

The continued production of almost 50% susceptible mice in the back-crosses is further evidence of the dominant one-locus explanation of genetic susceptibility to this agent. Incomplete penetrance may also be present in 8 and 9 week old mice of the C_3HSS strain since there was a sharp decrease in susceptibility of these mice even though their macrophages in culture maintained full susceptibility.

We wish to thank Dr. Ilan Shif for his contribution in testing for susceptibility.

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s on the Blood Clotting and Fibrinolytic System in the Plasma from a Sei (Baleen) Whale[1] (39427)

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ales are large deep-diving sea mam-
t belong to the suborder baleen
f the order Cetacea. They are said
le to dive to depths of 300 m or
d to stay under the water for rela-
g periods of time (1). As compared
estrial mammals, Cetaceans have
atomical and functional deviations
d with their aquatic life. Although
nparative studies of blood coagula-
and animals have been published
information is available concern-
cea. Robinson *et al.* (3) and Lewis
reported that the plasmas of the
bottle-nosed dolphin and killer
ich belong to the other suborder of
cea, the toothed whales, are defi-
Hageman factor (factor XII) activ-
e we have demonstrated a defi-
Fletcher factor (a plasma prekalli-
tivity as well (5). We have been
o find a description of the blood
on system in baleen whales. The
eport describes the studies of blood
and fibrinolytic systems in the
f one baleen whale species, the sei
alaenoptera borealis).

als and methods. Blood was col-
Iceland waters from a male sei
heart puncture 10–15 min after the
n of the animal. The blood was
the deck of a whaling boat with the aid of a vacuum pump. The anticoagulant used was 1/10 vol of citrate buffer (pH 5.0, 0.13 *M* with respect to citrate). The plasma was separated by centrifugation of the blood (1000 rpm, 10 min) immediately after the sampling. The plasma was frozen aboard the whaling boat and shipped to Cleveland in dry ice.

Pooled normal human plasma and plasma from patients with congenital clotting factor deficiencies were prepared as described earlier (6). Fletcher trait plasma and Fitzgerald trait plasma were kindly supplied by Dr. Charles Abildgaard, University of California at Davis, and Dr. Robert Waldmann, Henry Ford Hospital, Detroit, Mich., respectively.

Crude bovine thrombin (Topical Thrombin) and *Bothrops atrox* venom (Reptilase) were obtained from Parke, Davis & Co., Detroit, MI and Abbott Laboratories, Chicago, Ill., respectively. Rabbit brain tissue thromboplastin (Permaplastin) was purchased from Alban & Co., St. Louis, Mo. and human brain tissue thromboplastin from Cappel Laboratories, Wayne, Pa. Rabbit antiserum against human clotting factors were prepared as described earlier (6–8). Goat antiserum against human kininogen and human C1 esterase (C$\bar{1}$) inhibitor were kindly supplied by Dr. Virginia Donaldson, University of Cincinnati College of Medicine, Cincinnati, Ohio and Dr. Jack Pensky, Case Western Reserve University, Cleveland, Ohio. The following antiserums were obtained from commercial sources: rabbit antiserum against human plasminogen, fibrin-stabilizing factor (factor XIII) and inter-α-trypsin inhibitor from Behringwerke AG, Marburg, Germany; goat antiserum against α_2-macroglobulin and α_1-trypsin inhibitor from Miles Laboratories, Elkhart, Ind.; rabbit antiserum

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Investigator of the American Heart Associ-

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against fibrin from Hyland Laboratories, Costa Mesa, Calif.; and rabbit antiserum against antithrombin III from Nyegaard & Co. AS, Oslo, Norway.

Routine clotting tests and assays for individual clotting factor activity were performed as described previously (9, 10). One unit of clotting factor activity was arbitrarily defined as that present in 1 ml of pooled normal human plasma (6). Antithrombin activity (11), plasma inhibitor against activated PTA (12), and plasma inhibitor against activated Stuart factor (13) were measured as described. The inhibition of the clot-promoting activity of activated Fletcher factor (a plasma kallikrein) was tested by incubating purified plasma kallikrein (25 μM MeOH released/ml/hr in a TAME (*p*-toluene sulfonyl-L-arginine methyl ester) esterase assay (8), with equal amounts of sei whale plasma or bovine serum albumin (1 mg/ml in barbital–saline buffer, pH 7.4) at 37°. At intervals, aliquots were tested for residual activated Fletcher factor activity upon Fletcher trait plasma as a substrate. The results were expressed as percent of the initial clot-promoting activity.

A crude fibrinogen fraction (fraction I-0) was isolated from citrated plasma by the method of Blombäck (14).

Plasma plasminogen concentration was assayed by the method of Remmert and Cohen (15) after activation by streptokinase (high purity, the gift of Lederle Laboratories, American Cyanamide Co., Pearl River, N.Y.) or human urokinase (WHO Standard from Abbott Laboratories. a gift of Dr. A. Johnson, New York University Medical Center, N.Y.).

Double immunodiffusion studies were carried out on 7.5 × 2.5 cm glass slides in 0.9% agarose gel in barbital buffer (0.05 *M* sodium barbital, pH 8.4). Ten microliter samples were applied to the wells which were 3 mm apart. Precipitin lines were allowed to develop for 24 hr at room temperature. Immunoelectrophoresis was performed on glass slides of the same size at a constant voltage of 150 V for 60 min at room temperature.

Antibody neutralization tests for AHF were performed as recently reported (16).

Results. Blood clotting system. Routine clotting tests showed that, compared to human pooled plasma, the thrombin time, Reptilase time, and one-stage prothrombin time were moderately prolonged. The kaolin-activated partial thromboplastin time (kaolin PTT) was greatly prolonged (Table I). A crude fibrinogen fraction (fraction I-0), isolated from whale plasma, clotted more rapidly upon addition of thrombin than the corresponding fraction of human plasma. The one-stage prothrombin time of the whale plasma, using human tissue thromboplastin, was comparable to that in which rabbit tissue thromboplastin was used. The prolonged kaolin PTT (355 sec) was largely corrected by addition of an equal volume of human plasma (81.3 sec), suggesting that this prolongation was not due to the presence of a circulating anticoagulant in the whale plasma. Individual clotting factors were measured in comparison to pooled normal human plasma using human, congenitally deficient plasmas as substrates (Table II). Activities attributable to Hageman factor, Fletcher factor, and PTA were absent, whereas those of other clotting factors were comparable to those of human plasma. In immunologic studies employing double diffusion, whale plasma formed a precipitin line of nonidentity with human plasma against rabbit antihuman fibrin serum. Little difference was observed on immunoelectrophoresis between the mobility of the fibrinogen-related antigen of whale and human plasma. In contrast to fibrinogen, no precipitin lines formed between whale plasma and antiserums to human AHF, PTA, HF, Fletcher factor, Fitzgerald factor, and fibrin-stabilizing factor. AHF activity of whale plasma was also studied by antibody neutralization tests, using both rabbit anti-human AHF serum and a human circulating anticoagulant against AHF. Whale AHF was 7.5× more resistant to inactivation by rabbit antiserum and 7.6× more resistant to inactivation by the human circulating anticoagulant than was the AHF in human plasma.

Fibrinolytic system. When human urokinase was used as an activator, plasma plasminogen was detected at a concentration of 1.4 R and C units/ml, but not when streptokinase was used. Whale plasma formed a e precipitin line of nonidentity with human plasma against rabbit anti-human plas- gen serum. A precipitin line formed immunoelectrophoresis of whale na against this antiserum with elec- oretic mobility similar to that of an plasma.

hibitors of blood coagulation and fibri- is. Functional assays of plasma anti- nbin and plasma antiplasmin activity ed 200 and 50% activity relative to of a standard pool of normal human na, respectively. The titers of the inhib- against activated PTA and activated rt factor in whale plasma were 74 and % of normal human pooled plasma, respectively. When activated Fletcher factor (a plasma kallikrein) was incubated with baleen whale plasma, clot-promoting activity was decreased to 44% at 5 min and 26% at 10 min of the initial activity. There was, however, no loss of activity when activated Fletcher factor was incubated with a bovine serum albumin control. Immunodiffusion studies of whale plasma revealed single precipitin lines of partial identity with human plasma against the antiserums to human α_1-trypsin inhibitor, antithrombin III and C$\bar{1}$ inhibitor. Upon immunoelectrophoresis, little difference was observed between the mobility of human and whale plasma α_2-macroglobulin and inter-α-trypsin inhibitor-related antigen.

BLE I. ROUTINE CLOTTING STUDIES OF SEI WHALE PLASMA

	Clotting time (sec)		
Tests	Baleen whale	Human control	Whale + human (1:1)
bin time[a]	25.3	13.2	16.0
ase time	24.1	18.4	–
ombin time, brain ssue thromboplas- n			
nan	16.6	10.7	10.7
bit	19.6	13.3	–
-activated PTT	355.0	62.2	81.3

ive units per milliliter of bovine thrombin was

Discussion. Sei whale plasma showed a greatly prolonged kaolin PTT that was corrected by addition of normal human plasma. These experiments suggest that a deficiency of one or more clotting factors participating in the intrinsic pathway of thrombin formation was present. The most remarkable findings were the absence of detectable Hageman factor, Fletcher factor, and PTA activity. These results, in test systems comprising heterogeneous protein mixtures, should be interpreted carefully. A failure to correct the clotting defect in human Hageman trait, Fletcher trait, and PTA deficiency may have several causes. The factor may be absent in both plasmas, or it may function only in a

TABLE II. CLOTTING FACTOR ACTIVITY AND ANTIGEN IN SEI WHALE PLASMA

Clotting factors	Normal human range[a]	Activity (U/ml)	Precipitin line with heterologous anti-human clotting factor serum
Factor I (fibrinogen)[b]	177–415	141	+
Factor II (prothrombin)	0.65–1.50	0.90	NT[c]
Factor V (proaccelerin)	0.45–1.65	0.54	NT
Factor VII	0.55–1.85	0.17	NT
Factor VIII (AHF)	0.46–1.75	1.44	–
Factor IX (Christmas factor)	0.50–1.80	2.30	NT
Factor X (Stuart factor)	0.65–1.85	0.75	NT
Factor XI (PTA)	0.50–1.56	0.04	–
Factor XII (Hageman factor)	0.35–1.83	<0.01	–
Factor XIII (FSF)	Present	Present	–
Fletcher factor (plasma prekallikrein)	0.50–1.50	<0.01	–
Fitzgerald factor (high MW kininogen)	0.46–1.32	0.30	–

lean ± 2 SD.

xpressed as milligrams of protein per 100 ml of plasma.

ot tested.

species specific manner. Nonetheless, this combination of clotting factor deficiencies and the prolonged kaolin PTT suggest that the sei whale is truly deficient in these factors. In contrast, all other known clotting factor activities were present in whale plasma in amounts comparable to those present in human plasma. Although factor VII activity was moderately low, species specificity of tissue thromboplastin used to assay this factor may explain the low level (17). Immunological studies showed that the AHF, fibrin-stabilizing factor and Fitzgerald factor (high molecular weight kininogen) of whale plasma were antigenically different from those of human plasma. Whale fibrinogen, however, appeared to contain antigens partially similar to those of human fibrinogen.

• It is interesting to note that 3 out of 4 surface-mediated clotting factors (HF, PTA, Fletcher factor and Fitzgerald factor) were absent in the plasma of a deep-diving animal. The sei whale is said to dive as deep as 300 m or more (1). At these depths the water pressure is about 30 or more atmospheres. It has been shown that the blood circulation to some parts of the seal may be stopped or retarded during deep dives (18). The role of retarded blood flow in thrombosis is well appreciated (19). It is also said that blood pH falls during long dives (20). Acidosis may contribute to thrombogenesis (21). Yet these animals do not apparently undergo thrombosis. It is tempting to speculate that the deficiency of HF, Fletcher factor, and PTA may contribute to the lack of thrombosis in these deep-diving animals.

Normal human plasma possesses various protease inhibitors which may counterbalance the activation of blood clotting. Whale plasma appears to have similar inhibitors which can be detected functionally and immunologically. It is interesting to note that whale plasma is able to inactivate activated Fletcher factor and activated PTA, although both Fletcher factor and PTA are deficient in the plasma.

Studies on the fibrinolytic system in marine mammals are also sparse. The present study demonstrated that plasma plasminogen of a baleen whale was activated by human urokinase, but not by streptokinase. This is consistent with the previous report that plasma plasminogen in the killer whale and dolphins was not activated by streptokinase alone (3). The presence of plasma plasminogen in the sei whale was also demonstrated immunologically. Additionally, whale plasma had some antiplasmin activity.

Comparative study of blood coagulation and fibrinolytic systems in mammals may help not only to elucidate the evolution of these systems but also to understand the possible role of these systems in the pathophysiology of human disease.

Summary. Blood clotting and fibrinolytic systems were studied in the plasma of a sei whale (*Balaenoptera borealis*). The sei whale belongs to the suborder baleen whales of the order Cetacea. Whale plasma had a greatly prolonged kaolin-activated partial thromboplastin time and was deficient in Hageman factor (factor XII), Fletcher factor (a plasma prekallikrein), and PTA (factor XI). All other clotting factor activities were present in amounts comparable to that of normal human plasma. Whale plasminogen was activated by human urokinase, but not by streptokinase. Whale plasma contained inhibitory activities against thrombin, activated Stuart factor, activated PTA, activated Fletcher factor, and plasmin.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 508–511 (1976)

Carrier-Determined Suppression of Anti-fluorescein Antibody in the Rabbit (39428)

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Immunologic tolerance to simple hapten bound to nonimmunogenic carriers has gained wide acceptance to study the mechanism of tolerance (1–6). Of particular interest is autologous IgG as a tolerance-inducing carrier because it is a physiologic protein naturally tolerated by the host (5). Although this phenomenon has been observed in several strains of mice (7), it is not known whether this is a general property of autologous IgG. For this reason, we have examined the tolerogenic properties of autologous IgG in a different system, the anti-fluorescein response of rabbits (8). This particular system was chosen for two reasons. Firstly, the rabbit anti-fluorescein (anti-Fl) response to an immunogenic hapten carrier conjugate (Fl-KLH) (Fl-keyhole limpet haemocyanin) has already been extensively characterized in this laboratory (8). Secondly, if one can induced tolerance to a fluorescent hapten it will offer distinct advantages to follow the fate of the tolerogen in vivo.

Materials and methods. Preparation of hapten carrier conjugates. Keyhole limpet haemocyanin (KLH) and purified isologous rabbit gamma globulin (RGG) were obtained commercially from Schwartz-Mann. Autologous RGG was purified from the serum of individual rabbits by starch block electrophoresis. Fluorescein isothiocyanate (Sigma Chemical Co., St. Louis, Mo.) was conjugated in different molar ratios to KLH, RGG (homologous), and RGG (autologous) by previously described methods (8).

Immunizations. Randomly bred New Zealand white rabbits (Gloucester Rabbitry) were used throughout these studies. The rabbits were injected subcutaneously (in the rear footpads) with 3–5 mg of hapten carrier conjugate in complete Freund's adjuvant (CFA), or intravenously (in the marginal ear vein) with 30–50 mg of the hapten carrier conjugate in phosphate-buffered saline (PBS). For iv injections the molar ratio of hapten to carrier was 8–10:1 based on molecular weights of 900,000 for KLH and 150,000 for RGG. For sc injections the molar ratios were 85:1 for Fl-KLH and 8:1 for Fl-RGG. In some experiments iv and sc injections were performed simultaneously.

Anti-Fl assays. In these studies, the relative levels of anti-Fl responses to different hapten carrier conjugates and different methods of immunization were compared in terms of the maximum concentration of anti-Fl antibody reached in the serum following each injection.

The rabbits were bled weekly (35–40 ml) and screened for anti-Fl activity by qualitative precipitin tests with Fl-bovine serum albumin (BSA). (8) The RGG fraction in each serum sample was precipitated by $(NH_4)_2SO_4$ (30% w/w), dissolved in PBS (25 ml) and dialyzed against PBS to remove any traces of $(NH_2)_2SO_4$. The RGG solutions were then diluted to 0.5–1.0 mg/ml and assayed by fluorescence titrations (9) to determine the anti-Fl concentration. The actual concentration of anti-Fl in the original serum was calculated from this data. Using this technique, serum antifluorescein levels as low as 0.1 mg/ml could be detected.

Anti-KLH assay. The relative concentrations of anti-KLH antibody were determined by a quantitative precipitin test in which the precipitated antigen–antibody complex was assayed spectrophotometrically. The extinction coefficient ($E\pm^{\mathrm{cm/ml}}_{\mathrm{mg}}$) of IgG and KLH were assumed to be 1.5 (8) and 1.7 (10), respectively. The weight of anti-KLH antibody in the precipitate was then used to calculated the concentration (in milligram per milliliter) of anti-KLH anti-

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n the original serum.

lts. *Induction of immunological toler- Fl by Fl-RGG (autologous).* Previ- it has been shown that immunization he antigen Fl-KLH (sc) induces an response in rabbits. The maximum d within 2 weeks was in the range of g/ml (8) and is maintained for 2 to 3 then declines after 6 weeks. At this e serum anti-Fl concentration is less .1 mg/ml, the limit of detection in our The effect of treatment with Fl-RGG gous) on the anti-Fl response was . The results are shown in Table I.

nals (Group I) injected with Fl-RGG gous) iv had no detectable antibody their serum either prior to or after a iv injection of Fl$_8$-RGG (autologous) er with a subcutaneous injection of LH in CFA. In contrast, animals simi- eated with either Fl-RGG (homolo- (Group II) or Fl-KLH (Group III) e an anti-Fl response.

letermine whether pretreatment with ologous hapten-carrier conjugate was ary for the induction of tolerance, ntreated rabbits were injected, first l$_8$-RGG (autologous) iv, and then, iately thereafter, with Fl$_{85}$-KLH sc. ti-Fl antibody was detected in any of bits during the next 7 weeks.

ression of the secondary response to l-RGG (autologous). The results are in Table II. Six rabbits (Group IV) were immunized sc with Fl$_{85}$-KLH and bled weekly until the concentration of anti-Fl in the serum was less than 0.1 mg/ml. During this period, (6 to 8 weeks) the concentration of anti-Fl in the sera reached maximum levels of 1-2 mg/ml.

Each of the rabbits was then injected simultaneously with Fl$_8$-RGG (autologous) and Fl$_{85}$-KLH sc. Five of the six rabbits produced no detectable anti-Fl antibody during the next 6 weeks, and, in the sixth rabbit, the response was suppressed (0.3 mg/ml). In a control experiment, five rabbits similarly treated with Fl$_{85}$-KLH (Group V) showed a normal anti-Fl response (1-2 mg/ml) to both primary and secondary injections.

Seven weeks after the tolerizing injection, each of the tolerized rabbits were challenged with sc injection of Fl$_{85}$-KLH. In each case, the normal anti-Fl response was restored.

Table I and II show that the suppression of the fluorescein response is hapten specific since the immune response to the carrier (KLH) is the same in control rabbits as well as in rabbits made tolerant to fluorescein.

Immunogenicity of all Fl Carrier Conjugates. The results show (Table III), as expected, that Fl-KLH is more immunogenic than Fl-homologous RGG and that the subcutaneous route of administration is more effective than the intravenous route in inducing antibody formation. In contrast, Fl-

TABLE I. SUPPRESSION OF THE PRIMARY RESPONSE TO Fl BY Fl-RGG (AUTOLOGOUS)

No. of rabbits	Experimental schedule (week)	Conjugate	Route of administration	Average maximum anti-Fl concentration in serum (mg/ml)[a]	Average maximum anti-KLH concentration in serum (mg/ml)
5	0	Fl-RGG (Autologous)	IV	<0.1[b]	–
	8	Fl-RGG (Autologous)	IV	<0.1	0.73 (0.03)
		Fl-KLH	SC		
3	0	Fl-RGG (Isologous)	IV	.25 (0.05)	–
	8	Fl-RGG (Isologous)	IV	1.25 (0.20)	0.75 (0.05)
		Fl/KLH	SC		
3	0	Fl-KLH	IV	0.45 (0.1)	0.78 (0.04)
	8	Fl-KLH	IV	1.5 (0.35)	0.76 (0.05)
		Fl-KLH	SC		

h of these values is the arithmetic mean of the maximum, reached after 2 weeks in all instances, anti-Fl ations found during the anti-Fl response of each of the rabbits in a particular group. The standard s are indicated in parentheses.

limit of detection of anti-Fl antibody in our assay was 0.1 mg/ml.

TABLE II. SUPPRESSION OF THE SECONDARY RESPONSE TO Fl BY Fl-RGG (AUTOLOGOUS)

Group	No. of rabbits	Time of infection (week)	Conjugate	Route of administration	Average maximum anti-Fl concentration in serum (mg/ml)[a]	Average maximum anti-KLH concentration in serum (mg/ml)
IV	5	0	Fl-KLH	SC	1.2 (0.15)	0.74 (0.03)
		8	Fl-RGG (autologous)	IV	<0.1[b,c]	0.77 (0.02)
			Fl-KLH	SC		
		16	Fl-KLH	SC	1.15 (0.30)	0.82 (0.04)
V	5	0	Fl-KLH	SC	1.15 (0.20)	0.73 (0.04)
		8	Fl-KLH	IV	1.30 (0.35)	0.71 (0.03)
			Fl-KLH	SC		

[a] Each of these values is the arithmetic mean of the maximum, reached after two weeks in all cases, anti-Fl concentrations found during the anti-Fl response of each of the rabbits in a particular group. The standard deviations are indicated in parentheses.

[b] The limit of detection of anti-Fl antibody in our assay was 0.1 mg/ml.

[c] A sixth rabbit gave a detectable response to the second injection yielding a maximum anti-Fl concentration of 0.3 mg/ml.

TABLE III. RABBIT ANTI-Fl RESPONSES TO Fl-CARRIER CONJUGATES

No. of rabbits	Conjugate	Moles of Fl per mole of carrier	Route of administration	Average maximum anti-Fl concentration in serum (mg/ml)[a]
8	Fl-KLH	85:1	SC	1.2 (0.2)
3	Fl-RGG (isologous)	8:1	SC	0.25 (0.05)
3	Fl-RGG (autologous)	8:1	SC	<0.1[b]
3	Fl-KLH	10:1	IV	0.45 (0.1)
3	Fl-RGG (isologous)	8:1	IV	0.25 (0.05)
3	Fl-RGG (autologous)	8:1	IV	<0.1

[a] Each of these values is the arithmetic mean of the maximum anti-Fl concentration reached in all instances 2 weeks after immunization found during the anti-Fl response of each rabbit in a particular group. The standard deviations are indicated in parentheses.

[b] The limit of detection of anti-Fl antibody in our assay is 0.1 mg/ml.

autologous IgG was not immunogenic even when given subcutaneously in complete Freund's adjuvant.

Discussion. These studies show that, in rabbits, either the primary or the secondary anti-Fl response to Fl-KLH may be suppressed by treatment with a nonimmunogenic hapten carrier conjugate Fl-RGG (autologous). The induced tolerance is due to the autologous carrier and is hapten-specific. Recent work has shown that the autologous nature of IgG is not sufficient for tolerogenicity. The mode of attachment of the hapten to the carrier is also critical for tolerance induction (11). The efficiency of autologous IgG as a tolerance inducing carrier, as compared to hemologous IgG may be due to an allotypic difference of these rabbit gamma globulins. Thus, an immune response against a foreign allotype may have rendered Fl-isologous rabbit gamma globulin immunogenic.

The induction of carrier-determined tolerance in unprimed animals appears to involve a cellular mechanism in which the receptors of the antigen binding cells (ABC) are blocked by persistently bound tolerogen (12). The recent observation (13) that, in rats, fluorescein presented as tolerogen remains on the surface of the lymphoid cell, is consistent with this interpretation.

We have also found that tolerance may be induced in primed animals, which, presumably, have a greater number of antigen-binding cells (ABC) specific for fluorescein than in umprimed animals. This observation suggests that the ABC of the primed animals, which are known to process a greater number of specific receptors on their surface (14), have been paralyzed. The observation that carrier determined tolerance is transient and requires the constant presence of the tolerogen may imply that receptor blockade is reversible in vivo as was recently shown to be the case *in vitro* (15) when tolerance was broken. This mechanism may involve both T and B cells as has been demonstrated in a different system (16).

Finally, it should be emphasized that the ability to induce tolerance to a fluorescent hapten offers several distinct advantages: Firstly, it will permit direct visualization of the fate of the tolerogen *in vivo*. Thus, one can determine by a more simple technique than autoradiography, whether the tolerogen, in contrast to the immunogen, stays on the surface of the antigen binding cell. Secondly, receptor blockade of the anitigen binding cell by a fluorescent hapten will allow the separation of the tolerant cell (i.e., antigen binding cell with its receptor occupied by the tolerogen), by means of a cell sorter apparatus (17). If a relatively pure population of tolerant cells can be obtained then a number of studies on the function as well as the metabolism of these cells can be undertaken, thereby opening a rational approach to the cellular basis of tolerance.

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Isolation and Partial Characterization of a Major Basic Protein from Rat Eosinophil Granules[1] (39429)

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The eosinophil major basic protein (MBP) is a low molecular weight, highly basic material which constitutes over 50% of the protein content of the eosinophil granule (1). MBP has been isolated from the eosinophils of guinea pigs and humans (1, 2). The physiochemical analyses of the guinea pig MBP indicate that it has a molecular weight of 11,000, is rich in arginine (13%), and contains two free sulfhydryl groups, as well as two intrachain disulfide bonds (3). On standing, MBP readily aggregates presumably by oxidation of its two free sulfhydryl groups. As yet, no specific biological activity has been found for this protein.

The rat is a potentially useful species for the study of eosinophil function. For example, parasitic infections, most notably *Nippostrongylus brasiliensis*, have been well studied in the rat and evidence that the eosinophil plays a protective role in immunity to parasitic infections has been presented (4, 5). Further, an immunoglobulin E (IgE)-producing tumor has been found in the rat (6). Thus, the rat could serve as a useful model for the study of the relationship between IgE and eosinophils in immunity to parasitic infections and in allergic diseases. Because of these considerations we determined whether rat eosinophils possess a protein like the MBP found in the guinea pig and human, and here we report the isolation and characterization of such a protein.

Materials and methods. Eosinophil granules were prepared essentially as described previously for the guinea pig (7, 8). Briefly, the peritoneal cavities of rats were repeatedly lavaged at weekly intervals with 50 ml of sterile saline until an eosinophil rich population of cells was obtained (7, 8). These cells were pooled and washed once with 0.34 *M* sucrose, and a granule-rich fraction was prepared by extraction with 0.34 *M* sucrose. In this procedure, 2–5 × 10^8 cells were suspended in approximately 10 ml of sucrose, mixed vigorously by repetitively pipetting with a 10-ml serologic pipet, and centrifuged at 400*g* for 10 min to remove unbroken cells and nuclei. The sediment from the first extraction was extracted three more times in the same manner, and the supernates from these extractions were pooled and centrifuged at 15,000*g* for 20 min. The resulting pellet was extracted with 1.5 ml of 0.01 *N* HCl and chromatographed on a Sephadex G-50 column equilibrated with 0.01 *N* HCl. Eosinophil granule proteins were analyzed by polyacrylamide gel using an 8% gel containing 1% sodium dodecyl sulfate (SDS) according to the method of Fairbanks *et al.* (9). Gels were stained for protein with Coomassie blue (9). Isoelectrofocusing was performed using an LKB multiphor electrophoresis apparatus according to manufacturer's recommendations with a 2–10 pH gradient. Amino acid composition was performed as described by Spackman *et al.* (10), using a Beckman Model 119 amino acid analyzer. Samples of MBP were hydrolyzed for 24 and 48 hr in 6 *M* HCl. Cysteine content was determined after oxidizing the protein with performic acid as described by Moore (11). Eosinophil granules were fixed in 3% glutaraldehyde, embedded in Epon 812, and processed for electron microscopy according to standard procedures.

Results. Although rats tolerated repeated peritoneal lavaging and responded with an increased number of eosinophils in the lavage fluids, their response was quantita-

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different than that obtained with ı pigs (7). In spite of frequent lavaging, ımber of cells obtained from the rats ignificantly less than we could obtain the guinea pig. For example, on the ge a single lavage in the guinea pig d about 6 × 10^7 total leukocytes of approximately 45% were eosinophils. : rat, we normally obtained 1 × 10^7 of which 40% were eosinophils. Thus per animal basis there was a sixfold ence in the number of cells obtained eritoneal lavage. Because the small er of eosinophils obtained from a sin- vage yielded insufficient quantities of n for analysis, the rats were lavaged times a week instead of once a week. animals tolerated the more frequent ng well, and similar numbers of eosin- were obtained. However, extraction mixed preparation of cells from the neal cavity (40% eosinophils) yielded ranules and vanishingly small quanti- f protein. We attempted to alter the tion protocol by adding heparin (250 ml) to the sucrose solution because in has been shown to be important for traction of human eosinophil granules Ve also enriched the rat eosinophils by entation through a sodium ditrizoate on (final purity 80% eosinophils), but er modification nor their combination ved the yield of granules and protein the cells. Therefore, we increased the er of animals from 20 to 50 and by this larger population and by lavaging ekly intervals, we were able to obtain ient quantities of granules for study.

ls contained in the peritoneal lavage were osmotically disrupted with 0.34 crose, and the granules were pelleted ntrifugation at 15,000*g* for 20 min. examined under the electron micro- , this pellet contained free granules some cytoplasmic membranes but no ent nuclear debris. The granules in ellet were not in as high a state of as had been obtained with the guinea sinophils, but the contaminating cellu- ebris evidently did not affect subse- extraction procedures. The pelleted les from approximately 2 × 10^8 eosin- were solubilized in 1.5 ml of 0.01 *N* and analyzed by SDS polyacrylamide gel electrophoresis. The SDS gels showed a number of bands with a major band in a position which corresponded to the guinea pig major basic protein, i.e., with a molecular weight of approximately 11,000 (Fig. 1). The granule extract was chromatographed on a Sephadex G-50 column equilibrated with 0.01 *N* HCl and two major peaks were found, a void volume peak and a second peak eluting just after the cytochrome *c* marker indicating a molecular weight of around 11,000 (Fig. 2). When the material

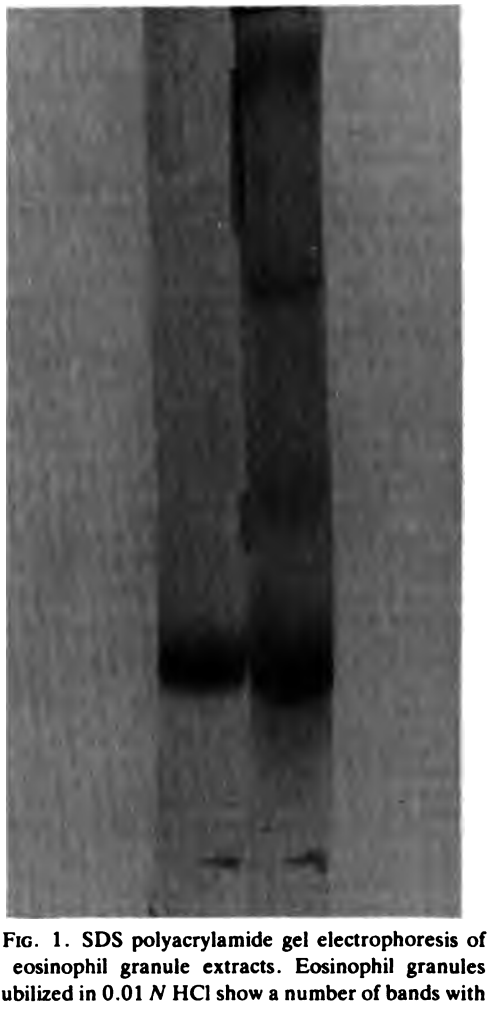

FIG. 1. SDS polyacrylamide gel electrophoresis of rat eosinophil granule extracts. Eosinophil granules solubilized in 0.01 *N* HCl show a number of bands with a major, low molecular weight band (right). Analysis of a concentrated pool of the second peak of a Sephadex G-50 column equilibrated with 0.01 *N* HCl reveals a single band at the same position as the major band (left). The india ink mark at the bottom of the gels indicates the position of the pyronin Y marker at the conclusion of electrophoresis.

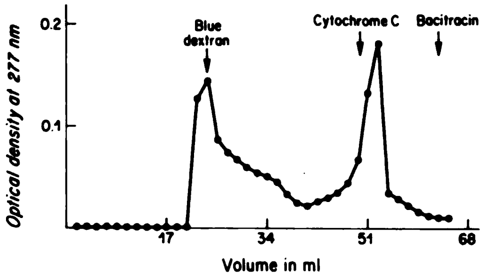

FIG. 2. Analysis of solubilized eosinophil granules on Sephadex G-50. Granules were solubilized in 0.01 *N* HCl and chromatographed on a 1.2 × 50 cm column equilibrated with 0.01 *N* HCl. The second peak fractions (elution volumes between 48–54 ml) were pooled, concentrated, and analyzed in the SDS-PAGE experiment shown in Fig. 1.

in the second peak was analyzed by SDS gel electrophoresis, it yielded a single band migrating in the position of the major band of the acid extract (Fig. 1). The protein from the second peak of the Sephadex G-50 column was analyzed by isoelectric focusing and a single band was seen at the cathodal wick indicating that the protein has an isoelectric point greater than 10. Amino acid composition of the eosinophil major basic protein was performed and the results are shown in Table I. The amino acid compositions of guinea pig and human MBP are shown for comparison. The amino acid compositions of the three proteins are quite similar for a number of amino acid residues and most significantly in their arginine content.

Discussion. Rats responded to repeated peritoneal lavage with sterile saline by developing a peritoneal eosinophilia. While the number of leukocytes obtained from the rat was less than the number obtained from guinea pigs by similar procedures, the yield was comparable to that reported by Bosworth and Archer (8). Surprisingly, we were unable to extract granules from cells obtained by frequent lavaging (three times per week) while granules could be obtained if the rats were only lavaged once a week. It is possible that the frequent lavaging selected a more immature population of eosinophils and the immature cells did not behave in the extraction protocol as expected from our prior studies.

TABLE I. AMINO ACID COMPOSITION OF MBP ISOLATED FROM RAT, GUINEA PIG, AND HUMAN

Amino acid	Rat	Guinea pig[d]	Human[e]
Aspartic	7[a]	6	7
Threonine	5	4	4
Serine	7	4	4
Glutamic	7	8	8
Proline	4	5	1
Glycine	14	12	8
Alanine	7	8	5
Valine	6	9	5
Methionine	3	1	0
Isoleucine	5	2	3
Leucine	5	4	6
Tyrosine	1	4	4
Phenylalanine	4	5	5
Histidine	2	3	2
Lysine	3	2	3
Arginine	13	13	11
Tryptophan	—[b]	4	8
Cysteine	5[c]	6	6

[a] Number of residues.
[b] Not done.
[c] As cysteic acid.
[d] From Ref. 3.
[e] From Ref. 2.

The rat eosinophil appears to contain a protein analogous to the MBP found in guinea pig and human eosinophil granules. This conclusion is based on three lines of evidence: (i) SDS polyacrylamide electrophoresis and gel filtration on Sephadex G-50 show a major band with a molecular weight of 10–12,000. (ii) Isoelectric focusing reveals that this protein has an isoelectric point greater than pH 10 indicating that it is highly basic. (iii) The amino acid composition data show that this is an arginine-rich protein. We concluded, therefore, that the rat eosinophil contains an MBP very similar to that isolated from the guinea pig and human. Although we did not obtain rat eosinophil granules in a high state of purity, the similarity of this protein to guinea pig MBP, which we have localized in the eosinophil granule core by immunoperoxidase electron microscopy (12), leads us to believe the rat MBP is derived from the eosinophil granule. The demonstration of MBP in rat eosinophil granules as well as our prior findings in the guinea pig and human suggests that this protein is common to most, if not all, mammalian eosinophils.

Summary. The rat eosinophil granule possesses a major basic protein (MBP) similar to that previously isolated from human and guinea pig eosinophils. This conclusion is based on demonstration that the major protein of the rat eosinophil granule is a low

molecular weight, highly basic material with an amino acid composition similar to that of the human and guinea pig MBP.

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A Radioimmunoassay for Serum Calcitonin in the Rat (39430)

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Despite extensive utilization of the rat to characterize the physiologic and pathophysiologic roles of calcitonin and factors which influence the secretion of the hormone, no radioimmunoassay has been developed with sufficient sensitivity to measure serum calcitonin in this species. Recently, Burford and associates (1) reported the important observation that human and rat calcitonin are closely related immunologically and that antisera to human calcitonin might be used to measure calcitonin in the rat. The hormone, however, could not be measured in the peripheral circulation with their assay. We now describe studies with a radioimmunoassay originally developed for human calcitonin (2, 3) which has sufficient sensitivity to permit characterization of calcitonin secretion in sera from normal rats.

Methods and materials. Male Sprague-Dawley rats weighing between 125 and 200 g were utilized. They were fasted overnight and blood samples were obtained by orbital puncture with the animals under light ether anesthesia for determination of calcium (4) and calcitonin. Thyroparathyroidectomized male Sprague-Dawley rats (Hormone Assay Laboratories, Inc.) weighing between 150 and 165 g were similarly studied. Sera from these animals were used for the radioimmunoassay. Calcium, 4 or 4.8 mg/100 g body wt, as calcium gluconate was given as a single intraperitoneal injection.

Thyroid glands were removed with the animals under ether anesthesia. The glands were freeze-dried and extracted three times with 1 ml of butanol:acetic acid:water (75:7.5:21) as previously described (5). The three extracts were combined, freeze-dried and dissolved in 1 ml of 0.6% acetic acid with 1% bovine serum albumin. The extracts were then serially diluted in the buffer for the radioimmunoassay.

Radioimmunoassay. The procedure described by Sizemore *et al.* (2) for radioimmunoassay of human calcitonin was employed with antiserum from chicken 513 (3). This was kindly supplied by Dr. Glen Sizemore. Serum from thyroparathyroidectomized rats was added to the buffer instead of human athyreotic plasma at a concentration of 5%. The buffer was 0.1 *M* tris(hydroxymethyl)aminomethane, pH 7.8, with kallikrein-trypsin inhibitor (Trasylol), 500 kIU/ml (FBA Pharmaceuticals, Inc.). The incubation volume was 0.5 ml and the final concentration of antiserum was 1:30,000 dilution. For each serum sample, incubations were carried out without antiserum to determine "damage" to the labeled hormone. Fifty microliters of serum samples were used for the assay which was carried out in triplicate. Human synthetic calcitonin (Ciba-Geigy Corp.) was labeled with ^{125}I by the method of Tashjian (6). About 15,000 cpm of ^{125}I-labeled calcitonin per tube was incubated. Bound and free [^{125}I]calcitonin were separated by dextran-charcoal (7). The sensitivity of the assay is 3 pg and corresponds to a lower detection limit of 60 pg/ml in serum. The coefficient of variation within assays was 22.6% and between assays was 32.6%. The same assay was used when the effects of calcium were examined for determination of basal and stimulated values.

Correlation coefficient was calculated with a 9810A Calculator (Hewlett-Packard).

Results. The radioimmunoassay curves with human calcitonin standard and extracts from thyroid glands of two rats and serum from a normal rat are shown in Figs. 1A and 1B, respectively. Based on these results the two glands were estimated to contain 100 and 250 ng of calcitonin, respectively.

Serum calcitonin was not detectable in each of five thyroparathyroidectomized rats even after calcium administration had brought the serum calcium to within the

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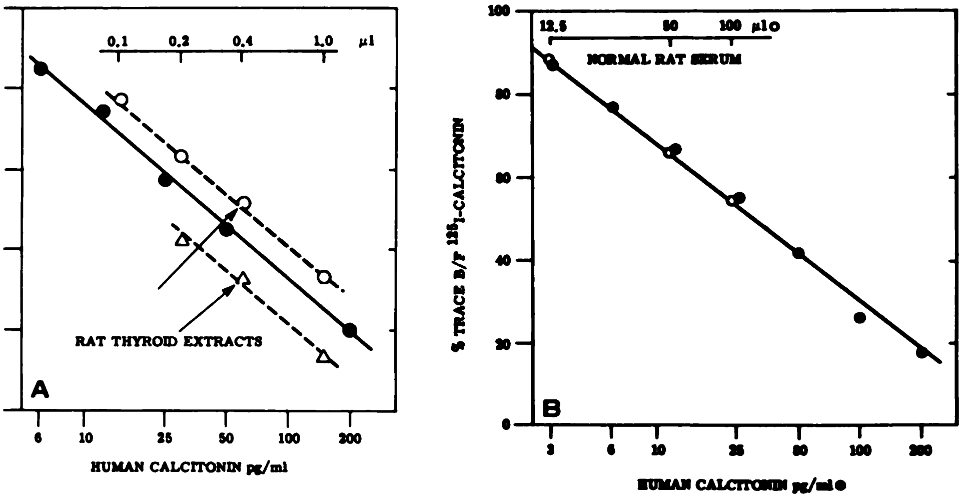

Immunoassay curve for ^{125}I-labeled human calcitonin with antiserum CK-513 against synthetic human . Displacement by unlabeled synthetic human calcitonin and by two thyroid extracts from normal rats ›y serum from a normal rat (B) are shown.

range or above (Fig. 2). In contrast, ılcitonin ranged from 71 to 175 pg/ even animals and serum calcitonin reased to a range of from 180 to 322 ›y 30 min after calcium administra- this study serum calcitonin was not 1 in one normal rat either before or lcium administration.

serum calcitonin ranged from below its of detection (<60 pg/ml) to 650 Mean serum calcitonin was compara- ıale and female rats both in the basal d after calcium, 4.8 mg/100 g body ›le I, Fig. 3).

relationship between serum calcium um calcitonin in the basal state and after the administration of calcium is n Fig. 3. The correlation coefficient significant in the fasting basal state .030, $p > 0.1$) but was statistically ınt when values 30 min after calcium tration were included ($r = 0.868, p$ 1).

ssion. The results of the present show that serum calcitonin changed hysiologically predictable manner. ıin was not detectable in sera from rathyroidectomized rats regardless her the rats were hypocalcemic or they had been given calcium and normal or high serum calcium val- contrast, increases in serum calcium produced by intraperitoneal administration of the ion produced increases in serum calcitonin in each of the normal animals in which basal levels of calcitonin could be measured. Finally, extracts of each of two thyroid glands from rats diluted out in a manner identical to that of the human calcitonin standard. These results provide strong evidence for the validity of the assay.

Previous studies in the rat have indicated that the parafollicular cells are apparently well adapted to secrete calcitonin in response to acute stimulation (9). Whereas chronic hypercalcemia leads to intrathyroidal depletion of the hormone, sustained hypocalcemia produces striking increases in the thyrocalcitonin content in the thyroid. In subjects with hypocalcemia, marked increases in calcitonin secretion are produced by calcium and pentagastrin (10). Thus, with hypocalcemia there is increased storage of the hormone. These studies provide evidence that calcitonin synthesis and secretion are poorly coordinated (9). We believe this lack of coordination accounts for the variation in serum calcitonin in the basal state and after calcium administration found in the present study and for the variation in release of calcitonin by pork thyroid slices in the basal state and in response to a variety of stimuli described in previous studies from our laboratory (11, 12). It also may be the

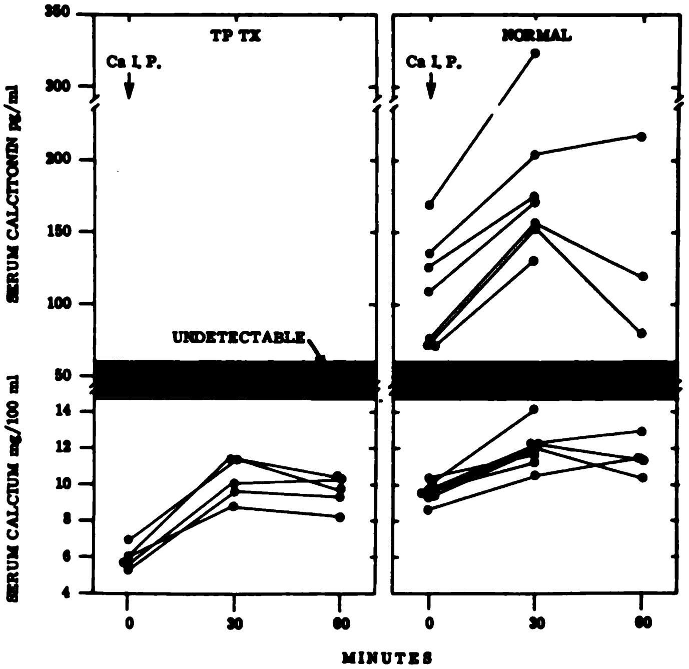

FIG. 2. The effects of intraperiotneal (ip) injection of calcium (Ca), 4 mg/100 g body wt, on serum calcitonin and serum calcium in five thyroparathyroidectomized (TPTX) and in eight normal male rats.

TABLE I. THE EFFECTS OF CALCIUM[a] ON SERUM CALCIUM AND SERUM CALCITONIN IN NORMAL MALE AND FEMALE RATS[b]

		Serum Calcium (mg/100 ml)			Serum Calcitonin (pg/ml)		
Sex	*N*	Control	30 min	*P* value	Control	30 min	*P* value
Male	7	9.5 ± 0.1	13.3 ± 0.7	<0.001	517 ± 30	2,271 ± 311	<0.001
Female	8	9.5 ± 0.1	14.1 ± 0.8	<0.001	417 ± 30	2,858 ± 175	<0.001
		NS	NS		NS	NS	

[a] 4.8 mg/100 g body wt ip.

[b] Results are given as means ± SE.

cause for the difference in quantity of calcitonin in extracts of rat thyroids observed in the present report as compared to previous studies (1) and for the inability of Talmadge and associates (13) to detect calcitonin in peripheral blood of normal Holtzman rats with a radioimmunoassay for rat calcitonin which has a lower limit of detection of 240 pg/ml. In this study, values as high as 600 pg/ml were noted during feeding when highly purified rat calcitonin was used as a standard. In subsequent studies, Cooper *et al.* (14) found that mean serum calcitonin was increased acutely to above 2,000 pg/ml after calcium was administered intravenously and to about 500 pg/ml after calcium was administered by mouth in Holtzman rats. Basal levels were not detected (<120–240 pg/ml). This difference in basal values as compared to our own may reflect strain differences, differences in the radioimmunoassay itself, or differences in physiological status of the animals at the time of study.

Serum calcitonin has previously been measured by bioassay in a system which utilized tissue culture of long bones of fetal rats labeled *in vivo* with ^{45}Ca (8). It was found that the concentration in serum from normal rats after parathyroidectomy was less than 0.05 MRC milliunit per milliliter (180 pg/ml). Thirty minutes after calcium administration, values as high as 1 to 2

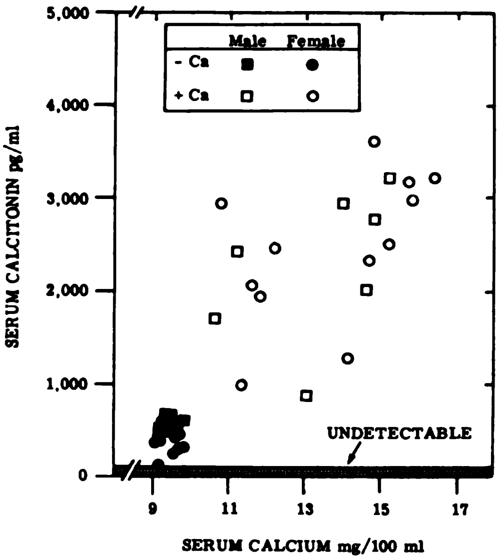

FIG. 3. Relationship between serum calcitonin and serum calcium in normal male rats given either no calcium (−Ca) or calcium (+Ca), 4.8 mg/100 g body wt 30 min previously. The correlation coefficient is significant if values after calcium are included (r = 0.868, $p < 0.001$) and is not significant for the unstimulated values (r = 0.030, $p > 0.1$).

MRC milliunits per milliliter (3600-7200 pg/ml) were found. These latter values are higher than those herein reported by radioimmunoassay. As noted already, there is evidence that calcitonin is stored in hypocalcemic states (9) and an exaggerated increase in serum calcitonin in response to calcium challenge has been reported (10). Therefore, the high values obtained by bioassay in the study just cited may reflect this phenomenon to some extent.

Summary. A radioimmunoassay for serum calcitonin in the rat is described. Materials for assay of human calcitonin are employed. The sensitivity of the assay is 3 pg and corresponds to a lower limit of detection of 60 pg/ml for peripheral serum. The normal range is up to 650 pg/ml in rats weighing between 125 and 200 g. Serum calcitonin is not detectable in thyroparathyroidectomized animals. It increases and varies directly with the serum calcium after intraperitoneal administration of the ion. Values for serum calcitonin in male and female rats are comparable under basal conditions and after calcium. The radioimmunoassay should allow the further characterization of calcitonin secretion in response to physiologic and pathophysiologic stimuli in this species.

These studies were supported by research funds from the Veterans Administration. The technical assistance of Mr. Jack Kimble and Mrs. Sarah Larson is gratefully acknowledged.

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Glutathione Peroxidase Activity and Glutathione Concentration in Genetically Dystrophic Mice[1] (39431)

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Dystrophia muscularis is a primary hereditary myopathy exhibited by the inbred strain of mice 129/ReJ-dy. This disease, which is characterized by progressive muscular weakness and atrophy of the skeletal muscles (1, 2), resembles muscular dystrophy in human beings in several aspects (3). Although the disease has been the subject of much investigation, its metabolic basis remains unknown.

It is well documented that dietary selenium deficiency can produce nutritional muscular dystrophy in a variety of species (4) and that the symptoms are similar to those seen in inherited muscular dystrophy. Recently it has been demonstrated that selenium functions as a component of glutathione peroxidase (glutathione: hydrogen peroxide oxidoreductase, EC 1.11.1.9) (5, 6) and that the activity of the enzyme decreases markedly in response to dietary selenium deficiency (7). Glutathione peroxidase evidently serves to protect the cell from lipid free radical damage by reducing fatty acid hydroperoxides to their correspondng hydroxy acids. It also functions in the removal of hydrogen peroxide generated during cellular metabolism (8).

In view of the important role of selenium in the prevention of nutritional muscular dystrophy and selenium's function as a part of glutathione peroxidase, the present study was conducted to determine whether inherited muscular dystrophy of mice is associated with any change in specific activity, substrate availability, or apparent K_m value of glutathione peroxidase in the muscle.

Materials and methods. Female dystrophic mice of the 129/ReJ-dy strain and their age-matched normal controls, 129/ReJ, were obtained from Jackson Laboratories[4] and maintained with stock rodent chow (Wayne Lab Blox). Animals were sacrificed in pairs over a 50-day period after signs of muscular dystrophy had become apparent. The mice were anesthetized with ether and a blood sample was obtained by heart puncture. The whole animal was perfused with saline by a saline drip into the heart in order to remove blood from the tissues. Heart, liver, lung, and skeletal muscle were removed and homogenized in 2 ml or 2 vol of buffer (0.01 *M* phosphate buffer containing 0.135 *M* KCl, pH 7.0). One sample of skeletal muscle was obtained from the hind legs and a second sample from the fore legs. The tissues were homogenized for 5 sec on a Polytron PT 10 homogenizer. Homogenates were centrifuged at 48,000*g* for 1 hr and the supernates were analyzed for glutathione peroxidase activity. Red blood cells were washed with saline and hemolyzed in deionized water before enzyme assay. Glutathione peroxidase activity was determined by the method of Little *et al.* (9). Oxidation of reduced NADP was measured spectrophotometrically at 340 nm in an assay medium containing 0.25 m*M* reduced glutathione (GSH), 0.12 m*M* NADPH, 0.1 *M* Tris-HCl buffer (pH 7.0), 3 m*M* EDTA, 0.2 m*M* cumene hydroperoxide, and excess glutathione reductase. All assays were carried out at 25°. These conditions resulted in minimum nonenzymatic activity. The protein content of the supernate was measured by the method of Lowry *et al.* (10). Glutathione peroxidase activity is expressed as nanomoles of reduced NADP oxidized per minute per milligram of protein.

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For glutathione analysis, the tissues were homogenized by the same procedure, and the concentration of GSH was measured by the method of Mills as modified by Hafeman *et al.* (7).

Results and Discussion. The dystrophic mice weighed significantly less ($P < 0.001$) than their age-matched controls at 40 days of age but showed few signs of dystrophy. During the ensuing 50 days, all dystrophic mice developed the symptoms of dystrophy previously described for this strain (1, 3) including kyphosis, paralysis of hind legs, periodic backward neck extension, denuded eyelids, and periocular inflammation.

Glutathione peroxidase was measured over a period of 7 weeks to determine whether enzyme activity changed as the skeletal muscles exhibited increasing degrees of paralysis (Figs. 1 and 2). Enzyme activity was higher in skeletal muscle of dystrophic mice than in that of normal mice (Table I). This increase was statistically significant ($P < 0.05$) for the muscle from the fore leg. Omaye and Tappel (11) recently reported that glutathione peroxidase activity is increased in skeletal muscle of the hind quarters of dystrophic mice of the 129/ReJ-dy strain. The hind legs exhibit signs of muscular weakness and paralysis earlier than the fore legs in this strain. It is of interest that in the present study the increase in enzyme activity was not confined to skeletal muscle showing severe symptoms of dystrophy but was at least as evident in the less affected muscles of the fore leg. No differences in enzyme activity were found in heart muscle or in the tissues of the liver, lung, or red blood cells (Table I).

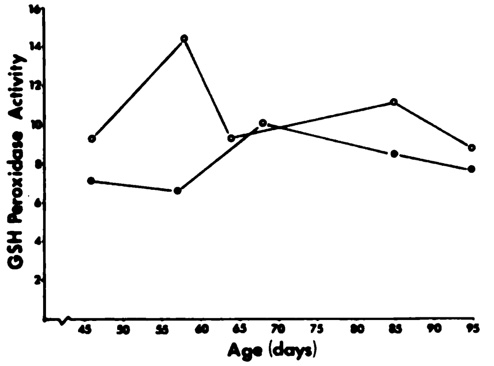

FIG. 1. Glutathione peroxidase activity in muscle from the hind legs of normal (●——●) and dystrophic (○——○) mice. Glutathione peroxidase activity is expressed as nanomoles of NADPH oxidized per minute per milligram of protein.

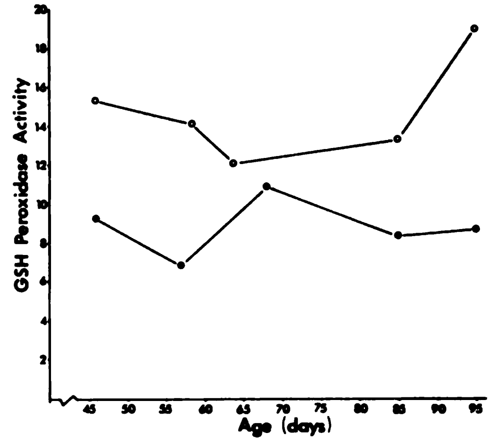

FIG. 2. Glutathione peroxidase activity in muscle from the fore legs of normal (●——●) and dystrophic (○——○) mice. Glutathione peroxidase activity is expressed as nanomoles of NADPH oxidized per minute per milligram of protein.

TABLE I. GLUTATHIONE PEROXIDASE ACTIVITY

	Nanomoles of NADPH oxidized/ min/mg protein	
Tissue	Normal	Dystrophic
Muscle (hind)	8.00 ± 1.04[a]	10.56 ± 0.61
Muscle (fore)	9.03 ± 0.83	14.77 ± 1.15[b]
Heart	18.48 ± 2.42	17.04 ± 1.13
Lung	100.63 ± 11.18	99.27 ± 8.36
Liver	394.50 ± 70.0	385.38 ± 66.70
Red blood cell	34.24 ± 5.14	27.77 ± 3.15

[a] Mean of five samples ± standard error.

[b] Significantly different from normals ($P < 0.05$).

Caution must be exercised in interpreting changes in enzyme specific activity based on protein concentration in dystrophic tissues. It has been demonstrated that differences between normal and dystrophic muscle in the concentration of enzymes and other constituents may reflect a change in the protein concentration of dystrophic muscle rather than a change in enzyme activity in the cells. The protein content of dystrophic mouse muscle may be only 25% of that found in normal muscle and all protein constituents are not reduced to the same extent. Many reported differences in enzyme activities between dystrophic and normal muscle can be attributed to secondary changes in muscle biochemistry which can be mimicked by denervation of normal muscle rather than to primary changes characterizing dystrophic muscle (12). Whether this is also true for glutathione peroxidase activity cannot be determined from the present or previous (11) studies. These studies do demonstrate, however, that muscular dystrophy in the 129/ReJ-dy mouse is not due to lack of glutathione peroxidase actvity in skeletal muscle or other tissues.

Since glutathione peroxidase is highly specific for GSH (13) and enzyme activity increases with increasing GSH concentration within the physiological range, levels of GSH were compared in various tissues from normal and dystrophic mice (Table II). GSH concentration was greater in skeletal muscle of dystrophic mice; this increase was statistically significant for the muscles of the hind legs. These data agree with previously reported values for GSH in skeletal muscle of normal and dystrophic mice (14). There were no differences between dystrophic and normal mice in GSH concentration in the heart, lungs, or liver.

Previous studies on glutathione peroxidase have shown that the kinetic behavior of the enzyme is not readily investigated due to the extremely low apparent K_m for H_2O_2 and the high velocity of the enzymatic reaction itself (9, 13). In addition, the apparent K_m for the hydroperoxide increases with increasing GSH concentrations. Utilizing the assay conditions previously outlined (with 0.25 m*M* GSH) apparent K_m values of 3.1 and 5.6 μ*M* cumene hydroperoxide were obtained for normal and dystrophic muscle, respectively. Apparent K_m values for liver enzyme for normal and dystrophic mice were 16 and 19 μ*M* cumene hydroperoxide. These values are valid only for the assay conditions described, but they serve to indicate that there were no detectable differences in the properties of glutathione peroxidase from normal and dystrophic muscle. The K_m values for the muscle enzyme are similar to those reported for glutathione peroxidase isolated from pig blood (9).

TABLE II. TISSUE GLUTATHIONE CONCENTRATION

	Milligrams of GSH per 100 g wet weight	
Tissue	Normal	Dystrophic
Muscle (Hind)	22.8 ± 0.9[a]	27.2 ± 1.6[b]
	(4)[c]	(3)
Muscle (Fore)	23.5 ± 2.3	29.5 ± 1.7
	(4)	(3)
Heart	36.8 ± 1.6	36.9 ± 4.2
	(4)	(3)
Lung	32.0 ± 3.4	31.2 ± 1.1
	(4)	(3)
Liver	190.2 ± 7.6	198.2 ± 8.6
	(8)	(7)

[a] Mean ± standard error.

[b] Significantly different from normals ($P < 0.05$).

[c] Entries in parentheses represent number of samples analyzed.

Summary. The present studies were conducted to determine whether inherited muscular dystrophy in the 129/ReJ-dy mouse was associated with differences in specific activity, substrate availability, or apparent K_m of glutathione peroxidase. The results indicate that glutathione peroxidase is elevated in skeletal muscle of mice with genetic muscular dystrophy when the activity is expressed on a protein basis. This elevation precedes the development of severe paralysis since muscles from the fore legs showed increased enzyme activity as early as the more severely affected hind legs. There was no difference in glutathione peroxidase activity in tissues other than skeletal muscle. GSH concentration was elevated in muscle and normal in other tissues of dystrophic mice, showing that adequate substrate was available to the enzyme. The apparent K_m for cumene hydroperoxide was also similar for muscle of normal and dystrophic mice. This report provides further evidence that mice with *dystrophia muscularis* have a functional glutathione peroxidase system in all tissues including skeletal muscle, and that a defect in this *in vivo* protective system is apparently not a contributing factor in the pathology of the disease.

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Seroepidemiologic Investigations of Human Hepatitis Caused by A, B, and a Possible Third Virus[1] (39432)

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The recent development of the immune adherence (IA) assay (1–3) for antibody against human hepatitis A together with established assays for hepatitis B antigen and antibody have made the conduct of seroepidemiologic investigations of these diseases possible. Evidence for a possible third agent other than type A or B and causing epidemic hepatitis in man has been presented (4). This report summarizes additional results of seroepidemiologic studies of hepatitis in human subjects in Costa Rica and in the United States.

Materials and methods. Human sera. The subjects in Costa Rica were residents of a community in which endemic hepatitis A and B occur and this has been under intensive epidemiologic investigation during the past 8 years (5, 6). The subjects in the United States were persons in the open community (Table I, Pennsylvania, high socioeconomic) and persons from selected populations (Table II), including plasmapheresis donors and employees or residents of institutions for the mentally retarded.

Serologic tests. The IA test for hepatitis A antibody was carried out as previously described by us (1), following the procedure of Mayumi *et al.* (7) as used for assays for hepatitis B antibody. A microtiter procedure was used, and each component was added in 0.025-ml vol. CR326 strain of hepatitis A virus, partially purified from the livers of infected *Saguinus mystax* (marmosets) served as antigen (1); 2–4 units were used in antibody assays. Complement was serum from Hartley-strain guinea pigs and was used at 1:50 to 1:100 dilution. Complement was stored at −70°. Human sera to be assayed for antibody were heated at 56° for 30 min and were assayed in serial twofold dilutions starting at a dilution of 1:5. The antigen–antibody mixtures were incubated at 37° for 1 hr. Complement was then added with thorough mixing. This mixture was further incubated at 37° for 40 min. Dithiothreitol solution (7) was then added to all wells with thorough mixing. This was followed by the addition of suitable human O, Rh-negative erythrocytes (1% suspension). This was thoroughly mixed and set for 3 hr at room temperature. Antibody titer was read as the highest dilution of serum (prior to addition of other reagents) at which complete erythrocyte agglutination was seen.

Tests for hepatitis B antigen and antibody and the biochemical tests for liver function were done as reported earlier (4). All sera were stored at −20° until assayed.

Results. Hepatitis A antibody distribution. The distribution of hepatitis A antibody, according to age, among persons in the open population in the United States and Costa Rica is shown in Table I. The Pennsylvania sera were from persons in high socioeconomic groups in the Philadelphia area who had been subjects in investigations of immune response to various vaccines other than hepatitis. The Costa Rican sera were from persons in the open community who were selected at random. It is seen that none of the persons in Pennsylvania who were less than 20 years of age had antibody against hepatitis A. Ten percent of the young adult group (20–29 years) had antibody, and this increased to approximately 40–50% in persons of middle age. By contrast, 20% of persons 1 year of age sampled in Costa Rica had hepatitis A antibody and this was increased to roughly 45% by age 2 to 3 years. By 10 years of age, 90% or more

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LE I. AGE DISTRIBUTION OF HEPATITIS A IA
TIBODY IN PERSONS IN OPEN POPULATIONS,
PENNSYLVANIA AND COSTA RICA

	Proportion seropositive			
Age	Pennsylvania		Costa Rica	
ears)	(No.)	(%)	(No.)	(%)
1	0/18	0	4/20	20
2	0/17	0	10/20	50
3	0/16	0	8/20	40
4-6	0/22	0	38/60	63
7-9	0/16	0	48/60	80
0-14	0/18	0	19/20	95
5-19	0/20	0	19/20	95
0-29	2/20	10	18/20	90
0-39	6/21	29	18/20	90
0-49	10/17	59	18/20	90
0+	5/12	42	16/20	80

BLE II. OCCURRENCE OF HEPATITIS A IA
NTIBODY AMONG PERSONS FROM SELECTED
POPULATIONS IN PENNSYLVANIA

		Serostatus	
Population	Age (years)	Positive/total	Positive (%)
esidents at Ham- State School and ital	21–51	9/10	90
yees at Laurelton School and Hos-	19–63	19/30	63
donors, Phila- ia	≥18	29/50	58
esidents at St. Jo- 's Home, Scran- and Hamburg School and Hos-	1–8	0/20	0

persons in Costa Rica had experience he hepatitis A virus.

presence or absence of antibody g persons in the United States was r investigated (Table II) in subjects were institutionalized, who were em- es of institutions, or who were paid rs of plasma. The highest rate for anti- presence was among adult residents of Iamburg State School and Hospital. t employees of a similar institution also ed a high frequency of previous experi- with the virus as did donors. None of ung children (1–8 years of age) in two tions for the mentally retarded had xperience with this agent.

nily epidemics of hepatitis, Costa Rica. III presents the results of investigations for hepatitis A and B in outbreaks in seven families in Costa Rica. Within the time periods shown, hepatitis A alone occurred in one family (033), hepatitis B alone in another (039), and both hepatitis A and B occurred in the five other families. Hepatitis A never occurred in persons with preexisting hepatitis A antibody, and hepatitis B did not occur in persons who had preexisting antibody against hepatitis B. Once introduced into a household, both hepatitis A and B appeared to be highly contagious, usually involving one to several susceptible members of the family within a period of a few months. Five cases of hepatitis occurred that did not develop antibody against hepatitis A or B virus and were possibly caused by agent(s) (hypothetical hepatitis C virus) other than the two known agents (4).

Table IV shows that the illness was clinically apparent in roughly half of the hepatitis A and B cases. The diagnosis in subclinical illness was based on significant elevations in transaminases and other biochemical determinations as well as antibody seroconversions. Four of the five cases of hepatitis in which serological evidence for hepatitis A and B was lacking showed subclinical disease and the fifth had anicteric hepatitis.

Table V shows that hepatitis A occurred in persons of very young age and that all but one of the 25 cases had occurred in persons less than 13 years of age (median age, 4 years). By contrast, hepatitis B occurred in persons of a wide range of ages, almost half being in persons 13 years or older. The cases of hepatitis other than A or B were distributed throughout the age spectrum. The difference in age distribution for hepatitis antibody in the families and the general population (Table I) was likely due to the fact that the family groups in whom epidemics occurred were necessarily selected for a high proportion of initially susceptible persons.

Discussion. Hepatitis A shows striking contrast in age distribution for antibody between persons in the open population groups that were studied in the United States and in Costa Rica. The chance for early infection of hepatitis A is high in Costa Rica and infection may occur in half the population by 2 years of age. By contrast, infection of half the population of high soci

TABLE III. Clinical and Laboratory Findings in Family Outbreaks of Hepatitis in Costa Rica

Family		Preexisting hepatitis antibody		Onset of hepatitis			
No.	Age of member	A	B	A	B	Other	Hepatitis
033	8	0	0	12/21/66	–	–	Clinical
	8	0	0	12/27/66	–	–	Subclinical
	9	0	0	1/5/67	–	–	Subclinical
	4	0	0	1/12/67	–	–	Subclinical
	2	0	0	1/12/67	–	–	Clinical
	11	0	0	2/1/67	–	–	Clinical
	10	0	0	2/7/67	–	–	Subclinical
	1	0	0	2/21/67	–	–	Subclinical
	48	+	0	–	–	–	–
	37	+	0	–	–	–	–
039	11	+	0	–	1/2/67	–	Clinical
	10	+	0	–	1/30/67	–	Clinical
	14	+	0	–	2/3/67	–	Clinical
	36	0	0	–	5/28/67	–	Clinical
	3	0	0	–	7/5/67	–	Subclinical
	33	+	0	–	/67-74	–	Subclinical[a]
	8	0	0	–	/67-74	–	Subclinical[a]
	9	+	0	–	–	–	–
	6	0	0	–	–	–	–
	4	0	0	–	–	–	–
	3	0	0	–	–	–	–
065	3	0	0	3/27/67	–	–	Clinical
	5	0	0	4/13/67	–	–	Subclinical
	1	0	0	4-5/67	–	–	Subclinical[a]
	39	+	0	–	6/8/67	–	Subclinical
	13	+	0	–	6/8/67	–	Subclinical
	32	+	0	–	–	–	–
	11	+	+	–	–	–	–
	9	+	+	–	–	–	–
	8	+	+	–	–	–	–
236	9	+	0	–	2/17/68	–	Clinical
	32	+	0	–	4/1/69	–	Clinical
	4	0	0	8/25/68	–	–	Clinical
	3	0	0	10/2/68	–	–	Subclinical
	28	+	0	–	–	–	–
	10	+	0	–	–	–	–
	7	+	0	–	–	–	–
	5	+	0	–	–	–	–
056	9	+	0	–	3/2/67	–	Clinical
	4	0	0	4/20/67	–	–	Clinical
	5	0	0	5/2/67	–	–	Subclinical
	37	0	0	/67-74	–	–	Subclinical[a]
	2	0	0	/67-74	–	–	Subclinical[a]
	40	+	0	–	–	–	–
	14	+	0	–	–	–	–
	13	+	0	–	–	–	–
	10	+	0	–	–	–	–
	7	+	+	–	–	–	–

TABLE III—*Continued*

Family		Preexisting hepatitis antibody		Onset of hepatitis			
No.	Age of member	A	B	A	B	Other	Hepatitis
330	9	0	0	4/6/68	–	–	Clinical
	7	0	0	5/9/68	–	–	Clinical
	12	0	0	5/13/68	–	–	Clinical
	6	0	0	6/3/68	–	–	Clinical
	2	0	0	6/17/68	–	–	Clinical
	6	+	0	–	2/15/69	–	Subclinical
	36	+	0	–	–	4/16/68	Subclinical
	16	+	0	–	–	4/16/68	Subclinical
	12	+	0	–	–	4/16/68	Clinical
	5	0	0	–	–	5/14/68	Subclinical
008	15	+	0	–	10/11/66	–	Clinical
	7	+	0	–	12/13/66	–	Subclinical
	6	+	0	–	8/28/67	–	Subclinical
	2	0	+	8/20/67	–	–	Clinical
	4	0	0	8/25/67	–	–	Clinical
	3	0	0	8/28/67	–	–	Subclinical
	2	0	+	–	–	11/15/66	Subclinical
	34	+	0	–	–	–	–
	29	+	0	–	–	–	–

[a] No illness reported. Assumed to be subclinical. Diagnosis based solely on seroconversions.

TABLE IV. SEVERITY OF ILLNESS IN HEPATITIS CASES (TABLE III)

	Hepatitis type (No. persons)		
Hepatitis	A	B	Non A, Non B
Clinically evident	13	8	1
Subclinical	12	8	4

TABLE V. DISTRIBUTION ACCORDING TO AGE OF HEPATITIS CASES (TABLE III)

	Hepatitis type (No. persons)		
Age (years)	A	B	Non A, Non B
1-3	9	1	1
4-6	7	2	1
7-9	5	4	0
10-12	3	2	1
13-16	0	3	1
32-39	1	4	1
Total	25	16	5
Median age	4.0	10.5	12.0

oeconomic level in the United States does not occur until middle age. It may well be that hepatitis A may occur at a younger age in low socioeconomic groups. Adult residents of institutions in the United States and their attendants show a far greater incidence of antibody against hepatitis A than do their counterparts in the open population. Additionally, paid volunteer donors of plasma in the United States (often persons of relatively low socioeconomic standing) also show a relatively high level of previous experience with the agent. Interestingly, young children sampled in two institutions in Pennsylvania were free of antibody against this agent.

The presence of circulating antibody against hepatitis A or B appeared to afford immunity against their homologous agents in studies in family epidemics in Costa Rica. Hepatitis A, known to be of greater epidemic potential than hepatitis B (8), occurred on the average in persons at a younger age than did hepatitis B. Once introduced into the family, hepatitis B and especially hepatitis A viruses appeared to be capable of substantial spread among susceptible members of the group. The disease tended to be very mild (subclinical) in roughly half the subjects and clinically apparent in the other half, whether the illness be due to hepatitis A or B. Four of five patients not classified as A or B exhibited subclinical disease; the fifth case was clinical but anicteric. Of these five patients, three had preexisting hepatitis A antibody and a fourth had preexisting hepatitis B antibody. None developed detectable hepatitis B antigenemia. None showed a boost in antibody titers against hepatitis A or B in convalescence. Additional information concerning these five cases tentatively classified as hepatitis C has been presented in Villarejos *et al.* (4), cases 1 and 4-7.

Summary. Seroepidemiologic studies were made of normal subjects in populations in the United States and Costa Rica and in family outbreaks of hepatitis in Costa Rica. Hepatitis A affected a majority of children of very young age in Costa Rica, while such experience in persons of high socioeconomic status in the United States did not occur before middle life. Persons of low socioeconomic status (paid plasma donors) and residents and attendants of institutions for the mentally retarded showed a far greater incidence of hepatitis A antibody than did their counterparts in the open community. Hepatitis A and B epidemics occurred in families in Costa Rica with rapid spread to other susceptible members of the group. The disease was clinically apparent in roughly half the cases, whether the responsible agent be hepatitis A or B. Five cases of nonhepatitis A or B (hypothetical hepatitis C) were found and all but one of them were subclinical.

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cts of Glucose or Insulin on Myocardial Performance in Endotoxin Shock[1] (39433)

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cardial dysfunction in endotoxin or shock has been observed in several including dog (1, 2) and man (3, 4). ecise mechanism for the failure has description although inadequate cor- perfusion has been implicated as a y determinant (5), and substrate defi- has been suggested or implied (6, 7). ct toxic action of endotoxin on the ·dium seems to have been excluded and a proposed adverse effect of a :ing myocardial depressant factor (8) : been confirmed (2).

ɔglycemia has been documented to n a significant role in the pathogen- ɜndotoxin and septic shock in animals and man (9, 14). Hypoinsulinemia :n reported in subhuman primate sep- ck by Cryer's group (15) and Hin- r *al.* (16) and in low output human hock by Clowes *et al.* (17). Diabetic- ıcose tolerance responses have been ɜd in septic shock patients by Gump ıers (18).

nt reports have suggested prominent f glucose and insulin in improving output in shock (6, 7, 17). Weissler ıers (19) have suggested that the ef- insulin on the hypoxic isolated per- at heart is to increase utilization of . Clowes *et al.* (17) suspected that insulin exerted a possible direct ben- action on the myocardium in septic Hiatt *et al.* (20) found that massive of insulin (2400–7500 units) pro- survival in dogs for 30 hr to 10 days after ligation of the circumflex branch of the left coronary artery, whereas nontreated control dogs died within 16 min. They proposed that insulin infusion suppressed premature ventricular contractions, ventricular tachycardia, and fibrillation and diminished myocardial injury and ischemia (20). Our recent work documented the development of severe hypoglycemia during the intermediate stages of endotoxin shock. Animals becoming markedly hypoglycemic (<35 mg%) died in shock, whereas those receiving intravenous infusions of 50% glucose survived (13).

It occurred to us that the progressively developing hypoglycemia might be related to the precipitation of myocardial dysfunction in endotoxin shock. The purpose of the present study was to evaluate this possibility and determine the relative roles of glucose and insulin in supporting cardiac function in shock. Results from this study suggest that both the development of myocardial dysfunction following endotoxin insult and its subsequent reversal by insulin occur independently from changes in blood glucose concentrations.

Methods. Description of isolated heart preparations. Experiments were carried out on 15 isolated working canine heart preparations exchanging blood with intact support animals, as previously described (1, 2). The right ventricle was bypassed following cannulation of the pulmonary artery, and coronary venous return was obtained from a large-bore cannula introduced into the right ventricle (1, 2). During an equilibrium period, arterial blood from the support animal was supplied to the test heart, with lungs inflated, and the preparation was stabilized at a mean aortic pressure of 100 mm Hg and a cardiac output of 76 ml/min/kg (based on the weight of the dog supplying the isolated heart). Coronary venous return and aortic

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served.

outflow from the isolated heart were collected separately, measured volumetrically, and returned to the support animal (1, 2). Intraventricular pressures, including left ventricular end diastolic pressure (LVEDP) were monitored via pressure transducers connected to a plastic cannula penetrating the apex of the left ventricle. Mean aortic pressure, pulmonary blood flow, and blood temperature were maintained constant. Cardiac power (grams·meters/second) and maximum positive (+) and negative (−) *dP/dt* (millimeters of Hg/second) were calculated as previously reported (1, 2). Blood pressure in the support animal and various parameters in the isolated heart were continuously monitored on a Sanborn recorder. Afterload was varied periodically in order to evaluate myocardial performance.

Description of glucose and insulin studies. Arterial blood glucose was determined with a Beckman glucose analyzer possessing an accuracy of ±3 mg%. Insulin (Iletin, Eli Lilly and Company, Indianapolis, Ind.) was either infused into the left atrium of the isolated heart or administered to the support animal via the blood reservoir. Experiments were divided into three groups of five studies each and were composed of an initial group receiving an LD_{100} of *Escherichia coli* endotoxin (Difco, Detroit). Endotoxin was administered intravenously to the support dog and simultaneously to the isolated heart (based on the weight of the animal supplying the heart) via the pulmonary artery. Hypertonic glucose, 50%, was infused in some experiments into the reservoir receiving blood from the isolated heart, at rates sufficient to maintain the coronary arterial glucose concentration constant. A second series was conducted in which endotoxin was administered, and, following demonstration of myocardial failure, insulin was infused continuously into the left atrium of the isolated heart for the duration of the study.

A final group of experiments was performed to determine the effects of insulin infusion on nonfailing hearts not treated with endotoxin. Insulin was infused into the left atrium at rates sufficient to sustain significantly improved myocardial performance, as indicated by decreased LVEDP and elevated *dP/dt* values. In approximately 1 hr following steady-state effects of insulin on heart performance, insulin infusion was stopped, the support dog was removed from the perfusion system, isolated lungs were ventilated with 20% O_2 and 5% CO_2, and the heart was monitored for approximately 1 hr. Insulin blood values in the isolated perfusion system were determined by radioimmunoassay (Phadebas insulin test. Pharmacia, Uppsala, Sweden).

Statistics were carried out utilizing a Student's *t* test.

Results. Table I presents results from five isolated perfused working left ventricles exchanging blood with intact support dogs. Following establishment of baseline heart performance values, an LD_{100} of endotoxin was administered to both heart and dog, and the arterial concentration of blood glucose reaching the heart was maintained constant by infusion of 50% glucose into the venous reservoir. Amounts of glucose infused varied greatly; however, the maximum decrease in glucose values from the control in all experiments did not exceed 4%. Results from individual heart studies shown in Table I demonstrate myocardial failure 3–4 hr postendotoxin: LVEDP rises significantly in each heart, and in heart No. 5, afterload could not be elevated above 50 mm Hg without induction of irreversible failure. Power values diminished from control in all instances while coronary blood flows were elevated in most hearts. Changes in positive and negative *dP/dt* and heart rate were variable. These results demonstrated that myocardial dysfunction after endotoxin may occur independently of arterial blood glucose concentrations, hypoglycemia being excluded, therefore, as a prerequisite for failure. The degree and time course of failure seen in the present series agrees with previously published reports (1, 2) on dogs subjected to endotoxin shock without glucose administration.

Table II illustrates results from a second series of five hearts receiving endotoxin as in Table I but additionally infused with insulin following the first significant indication of cardiac dysfunction as verified by imposed afterload stresses. The average time for demonstration of dysfunction was 3.4 hr postendotoxin as evidenced by significant

TABLE I. EFFECTS OF ENDOTOXIN ON MYOCARDIAL PERFORMANCE DURING GLUCOSE INFUSION[a]

Experiment	LVEDP[b] (mm Hg)		PE (hr)	Power (g·m/sec)		Coronary blood flow (ml/min/100 g)		$+dP/dt$ (mm Hg/sec)		$-dP/dt$ (mm Hg/sec)		Heart rate (min)	
	C	PE		C	PE	C	PE	C	PE	C	PE	C	PE
1	9.5	26.5	3	9.3	7.6	151	196	1613	1592	1645	1603	126	138
2	2.0	10.0	3	7.0	6.4	107	125	1923	1887	2632	2083	144	138
3	4.0	14.0	4	9.9	9.0	130	130	1851	1612	2173	2083	150	156
4	0.5	5.0	3	8.1	7.8	62	120	2000	1786	2778	2273	120	120
Mean ±SE	4.0 (2.0)	13.9 (4.6)		8.6 (0.6)	7.7 (0.5)	112 (19)	142 (18)	1847 (84)	1719 (71)	2307 (256)	2011 (143)	135 (7)	138 (7)
p Value	0.025												
5	4.0	17.5	4	4.3	3.1	69	96	862	922	1063	1136	120	114

[a] Infusion of 50% glucose at a rate sufficient to maintain arterial blood concentration constant. Expt 1–4 at afterload of 100 mm Hg; expt 5 at afterload of 50 mm Hg.

[b] LVEDP = left ventricular end diastolic pressure; C = control; PE = postendotoxin.

increases in LVEDP and depressions in power and $-dP/dt$ at 100 or 125 mm Hg afterloads. Mean glucose values declined, though insignificantly, during the postendotoxin period. Insulin administration was found to exert a beneficial action on myocardial performance: Infusion, which continued during the 3–5-hr period after endotoxin, was adjusted on the basis of achieving end diastolic pressures at control values or lower. Results demonstrate mean decreases in LVEDP at afterloads of both 100 and 125 mm Hg, within 2 hr of infusion, the most marked mean reduction, 17.1 to 4.3 mm Hg, occurring by the fourth hour at an afterload of 125 mm Hg. Coronary blood flow was increased and $+dP/dt$ was elevated at 100 mm Hg ($p \leq 0.025$). Changes in power, $-dP/dt$, and heart rate were insignificant in comparison with control values. Glucose concentration progressively fell during the postendotoxin course, reaching values of 43 and 33 mg% ($p < 0.05$) at afterloads of 100 and 125 mm Hg, respectively. These results demonstrated reversal of dysfunction by insulin infusion and sustained improvement of myocardial function during subsequent periods of hypoglycemia. Favorable effects of insulin were observed following its termination of infusion. Results from one experiment demonstrate its duration of action: Three hours following endotoxin administration, LVEDP rose from 3.0 to 22.5 mm Hg at an afterload of 125 mm Hg. Approximately 80 U of insulin was then infused during a period of 1.5 hr, resulting in a drop of LVEDP to 3.5 mm Hg. Insulin infusion was then terminated, and 50 min later LVEDP remained stabilized at 3.5 mm Hg at an afterload of 125 mm Hg.

The effects of insulin infusion on isolated hearts not receiving endotoxin are displayed in Table III. The first period of heart performance is 1–3 hr following onset of insulin administration, when the support dog is in the perfusion circuit (as in the previous series). The second and third periods of 3–4 hr represent the periods during which the heart was totally separated from the support animal so that there was no source of bloodborne agents. Results show notable decreases in LVEDP at 100 and 150 mm Hg

TABLE II. Effects of Insulin on Myocardial Performance in Endotoxin Shock[a]

Parameter	Control	Postendotoxin (mean, 3 hr)	Postinsulin (mean, 4 hr postendotoxin)	Postinsulin (mean, 5 hr postendotoxin)
Afterload, 100 mm Hg				
LVEDP (mm Hg)	4.9	9.3 (p, 0.02)	9.8	3.5
Power (g·m/sec)	11.2	10.6 (p, 0.01)	10.4	10.6
Coronary blood flow[b]	81	99	104 (p, 0.025)	120 (p, 0.02)
$+dP/dt$ (mm Hg/sec)	2132	2251	2293	3137 (p, 0.02)
$-dP/dt$ (mm Hg/sec)	2716	2028	2363	2771
Heart rate (min)	142	151	154	152
Glucose (mg%)	121	99	89	43 (p, 0.001)
Afterload, 125 mm Hg				
LVEDP	6.6	17.1 (p, 0.05)	4.3 (p, 0.05)	4.3
Power	13.9	12.4	14.2	14.2
Coronary blood flow[b]	100	117 (p, 0.05)	124	142
$+dP/dt$	2725	2230	2572	2997
$-dP/dt$	3302	2018 (p, 0.05)	2753	2929
Heart rate	146	150	146	149
Glucose	118	98	70	33 (p, 0.05)

[a] Data from five isolated canine heart preparations maintained at constant cardiac output and afterloads of 100 mm Hg (performance evaluated at 125 mm Hg). Blood is exchanged with intact animal. Ranges of insulin infused, 70–5500 units. Statistical significance compared to controls.

[b] Milliliters per minute per 100 g, left ventricle.

TABLE III. Effects of Insulin on Myocardial Performance in the Absence of Endotoxin[a]

Parameter	Control	Postinsulin (1-3 hr) (dog in circuit with heart)	Postinsulin (3.5 hr) (Heart isolated, dog removed)	Postinsulin (4.0 hr) (Heart isolated, dog removed)
Afterload, 100 mm Hg				
LVEDP (mm Hg)	2.7	0.7 (p, 0.02)	1.3 (p, 0.05)	1.4 (p, 0.02)
Power (g·m/sec)	9.3	9.5	9.4	9.4
Coronary blood flow[b]	103	118	114	114
$+dP/dt$ (mm Hg/sec)	2024	3321 (p, 0.001)	2970	2636 (p, 0.02)
$-dP/dt$ (mm Hg/sec)	2682	3448 (p, 0.01)	2983	2855
Heart rate (min)	140	154 (p, 0.05)	111 (p, 0.01)	104 (p, 0.001)
Glucose (mg%)	127	46 (p, 0.001)	28 (p, 0.001)	16 (p, 0.001)
Insulin infused (units)		7900	100	0
Afterload, 150 mm Hg				
LVEDP	4.5	1.4 (p, 0.02)	2.1	2.9
Power	14.0	14.2 (p, 0.05)	14.5	14.6
Coronary blood flow[b]	166	195	168	181
$+dP/dt$	2880	4936 (p, 0.005)	4113 (p, 0.025)	3983 (p, 0.05)
$-dP/dt$	3466	5020 (p, 0.02)	3895	4123 (p, 0.05)
Heart rate (min)	147	161 (p, 0.01)	114 (p, 0.025)	106 (p, 0.005)

[a] Data from five isolated canine heart preparations, receiving infusions of insulin without endotoxin. Statistical significances compared to control values.

[b] Milliliters per minute per 100 g, left ventricle.

afterloads ($p < 0.05$) within 3 hr after onset of insulin administration. Mean $+dP/dt$ increased while the arterial glucose level fell to 46 mg% ($p < 0.001$) within 3 hr of insulin infusion at an afterload of 100 mm Hg. During the same period, elevations of power, $+dP/dt$, and $-dP/dt$ were seen at 150 mm Hg ($p \leq 0.05$). Glucose values in arterial blood continued to fall after removal of the support animal from the heart perfusion system, reaching concentrations of 28 mg% at 3.5 hr and 16 mg% at 4.0 hr while LVEDP, power, coronary blood flow, $+dP/dt$, and $-dP/dt$ remained at control values or were improved above control values ($+dP/dt$, $p = 0.02$, at 100 mm Hg ıad and $+dP/dt$, $p = 0.025$, and $-dP/$ $= 0.05$, at 150 mm Hg afterload). ı concentrations in the reservoir at the f each experiment were in excess of U/ml. These observations suggest that ı infusion exerts a profoundly benefi- fect on myocardial performance which ained for long, steady-state periods ccurs in spite of extreme hypoglyce-

ussion. The purpose of this report) determine if the hypoglycemia of)xin shock bore a causal relationship elicitation of myocardial dysfunction. us work in this laboratory docu- d progressively developing cardiac (1) and lethal hypoglycemia (13) ndotoxin in the dog. It seemed rea- e that some connection might exist n the two pathophysiological phe- ıa. Administration of hypertonic glu- as been reported to elicit beneficial rdial responses and elevations of car- ıtput. Baue *et al.* (21) and Replogle hers (22) administered 50% glucose s in shock and reported increases in c output. Beneficial effects of glucose ically ill patients have been reported ıdyck's group (6) who observed in- s in cardiac output and left ventricular work with hypertonic glucose. These observations, including the reported lycemia in canine endotoxin shock, rvival benefits of infused glucose (9, endotoxin shock, and recent reports oglycemia in septic shock patients (9, trongly suggest that elevated blood e concentrations should augment my- al performance. Results from the t study, however, fail to confirm this ility: Severe myocardial dysfunction ed at all levels of glucose concentra- (5–125 mg%), and maintenance of e at control or higher levels by infu- as without benefit to the myocardium. findings suggest that the reported cial effects of infusion of hypertonic e may be due to peripheral rather lirect, cardiac actions. The observed se in cardiac output by Replogle's (22) and maintenance of mean arte- essure in dogs shocked with endotoxin ıay be due to augmented venous return and improved metabolic status following hypertonic glucose infusion.

Of particular interest in the present study were the significant beneficial cardiac effects of insulin in both failing and normal hearts. Very recently Weisul's group (7) demonstrated the effectiveness of an infusion of glucose, insulin, and potassium in clinical septic shock. They confirmed the presence of severe myocardial depression in low cardiac output septic shock and although isoproterenol improved performance it did not correct the depression of cardiac function. On the other hand, a solution of glucose, insulin, and potassium dramatically improved performance and corrected myocardial abnormalities (7). Weissler *et al.* (19) described the beneficial effects of insulin on the performance of the hypoxic isolated perfused rat heart and ascribed its benefit to the increased myocardial utilization of glucose. Weisul *et al.* (7) could not describe the mechanism of action of the solution of glucose, potassium, and insulin in their septic shock patients but suggested that it was linked to the prevention of both loss of potassium and gain of sodium in myocardial tissue, thus supporting the transmembrane action potential and myocardial contractility. Clowes and others (17) were also unable to explain the mechanism by which cardiac function is improved by glucose, potassium, and insulin in septic shock but suggested that glucose transport and glycolysis were enhanced and that the cell membrane potential was restored. Results from the present study indicate that insulin infusion alone clearly improves myocardial performance of the failing heart in endotoxin shock. Further, its strong inotropic influence did not depend on existing blood glucose levels, as was also observed in normal hearts not administered endotoxin. This observation is consistent with the view that insulin possesses a direct myocardial action, since normal hearts, which would not be expected to possess metabolic defects or altered transmembrane potentials, responded in a fashion similar to failing hearts. Of importance is the observation that severe hypoinsulinemia is consistently observed in subhuman septic shock (15, 16) and in patients with low output septic shock (17).

Since depressed heart function has been demonstrated in human septic shock (3, 4), the beneficial actions of infused insulin may be due in part to its important influences on myocardial contractility. Currently conducted isolated heart studies reveal an increase in heart rate from 158 to 169 beats/min ($p < 0.005$) at low doses of glucagon infusion (1 μg/min), and, since heart rates were unchanged after insulin in the present study, glucagon as an impurity in the insulin solution does not appear to be the agent mediating improvement of myocardial performance.

Summary. Past studies reported by this laboratory have documented myocardial dysfunction and progressively developing hypoglycemia in canine endotoxin shock. The purpose of the present study was to determine the effects of glucose concentrations and insulin infusions on myocardial performance following endotoxin administration. Experiments were carried out on isolated, working canine left ventricular heart preparations exchanging blood with intact dogs. Myocardial function was evaluated following endotoxin and correlated with concentrations of glucose and effects of insulin infusion. Cardiac dysfunction occurred within 2-4 hr postendotoxin and the degree of malfunction was not related to arterial blood glucose concentrations. Maintaining blood glucose at control, preshock, levels by infusion of 50% glucose did not prevent myocardial dysfunction as evidenced by elevations of left ventricular end diastolic pressure, and depressed power. Infusions of insulin reversed cardiac failure and maintained normal performance in spite of wide ranges in glucose concentration (5-120 mg%). Findings suggest that myocardial dysfunction is not precipitated or enhanced by the hypoglycemia of endotoxin shock. The beneficial actions of infused insulin on cardiac performance appear to be elicited on the basis of mechanisms other than myocardial glucose transport.

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' THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 535–539 (1976)

ic Relationships among Five Reovirus-Like (RVL) Agents by Complement (CF) and Development of New Substitute CF Antigens for the Human RVL Agent of Infantile Gastroenteritis (39434)

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ι we described the development of nent fixation (CF) test for the hu- irus-like (HRVL) agent of infan- oenteritis, using as antigen an ırticle-rich stool filtrate (1). Later ›ped a CF test for the serologically Nebraska calf diarrhea virus using concentrated cell culture- :DV as antigen (2). We also found CDV could be used as a substitute n for the HRVL agent as a large ı of individuals infected with the gent developed serological evi- infection not only to the human to the cell culture-propagated calf vell (2). Although the NCDV CF 'as not quite as efficient as the F antigen for detecting infection [RVL agent, the ready availability ılture-grown source of CF antigen rtant for epidemiological studies supply of the HRVL antigen was :cause particle-rich stools suitable ıntigen were found only infre- Ve also observed that the HRVL ; related by CF to the epizootic f infant mice (EDIM) virus (1, 2), ı agent (SA)-11, and the "O" (of- (2), which was recovered from :stinal washings of sheep and cat-

eport we describe: (i) the develop- CF test for the SA-11 and the "O" the antigenic relationships by CF ıe five reovirus-like agents in :tail since antisera to the SA-11 O" agent are now available; (iii) ıich demonstrate that the SA-11, EDIM viruses can also be used as CF antigens for the HRVL agent; tudies which reveal that the "O" agent, which grows quite readily in cell cultures, is significantly more efficient than the NCDV as a substitute CF antigen and should therefore be used preferentially when the HRVL agent is not available.

Materials and Methods. CF antigens for the HRVL agent and NCDV, prepared as previously described (1, 2), consisted of 2% stool filtrates containing the HRVL agent (D2, D2A, D3A, Da1A) and bovine embryonic kidney culture-grown 25× concentrated NCDV, respectively. The HRVL suspensions had a titer of 1–64 antigen units in tests with convalescent human sera. Three NCDV antigens had a titer of 1–8 units with convalescent NCDV calf sera; markedly higher titers resulted when 2 of the 3 antigens were also tested with hyperimmune NCDV guinea pig sera. A new lot of EDIM CF antigen which was higher titered (16–64 CF antigen units) than that previously used by us was purchased from Microbiological Associates, Inc.; the new lot was prepared from infected suckling mouse intestines using genetron treatment and centrifugation procedures (4). A CF test for the EDIM virus has been described previously (5). The HRVL agent, NCDV, and EDIM virus antigens were used undiluted in the CF tests to be described. Antisera for these three agents were prepared or obtained as previously described (2). The SA-11 and "O" viruses which had previously been passaged in vervet monkey kidney (MK) cell cultures and an additional six times in primary baboon kidney cell cultures were kindly furnished by Dr. H. H. Malherbe of the Southwest Foundation for Research and Education, San Antonio, Texas. These agents were further passaged in our laboratory in primary vervet (African

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green monkey) MK cell cultures maintained with equal amounts of Eagle's medium 2 and medium 199 with glutamine and appropriate antibiotics; serum was not used in the maintenance medium, and cultures were washed once prior to addition of maintenance medium to reduce the amount of residual serum from the growth medium. The "O" and SA-11 viruses induced easily detectable, definite, and reproducible cytopathic effect (CPE) by the fourth day after inoculation. The SA-11 and the "O" agent CF antigens used in this study were prepared as follows: The SA-11 and the "O" agent were passaged in vervet MK cell cultures in our laboratory a total of six and three times, respectively. On the fourth day after inoculation, when CPE was observed over most of the cell sheet, the cultures were frozen. Later the cultures were thawed and shaken on a Vortex mixer, and fluid and cells were harvested and used undiluted as CF antigen. The SA-11 and "O" viruses had infectivity titers of $10^{5.0}$ and $10^{5.5}$ $TCID_{50}$/0.2 ml, respectively. In tests with homologous hyperimmune animal sera the SA-11 and "O" viruses had CF antigen titers of 4-16 and 8-64 units, respectively. Antisera to the SA-11 and the "O" agent were prepared as follows: The SA-11 and the "O" agent were banded on a CsCl density gradient. Based on the knowledge that the serologically related NCDV and HRVL agent had a buoyant density in CsCl of approximately 1.35-1.37 g/cm^3 (2, 6, 7), we combined fractions with a density of 1.371 and 1.352 g/cm^3 from the SA-11 gradient and fractions with a density of 1.357 and 1.335 g/cm^3 from the "O" agent gradient. Each combined fraction was mixed with an equal volume of Freund's complete adjuvant, and 1.0 ml was inoculated intramuscularly into guinea pigs. About 4 weeks later, each combined fraction was mixed with an equal volume of incomplete Freund's adjuvant and 1.0 ml inoculated intramuscularly as a booster. The following control preparations were used in the CF tests to be described in Table I: 2% human stool filtrate, negative for the HRVL agent, prepared from a stool of an infant hospitalized with a respiratory tract illness; uninoculated bovine embryonic kidney cell cultures 25× concentrated; genetron-treated, centrifuged intestines from uninoculated suckling mice; and uninoculated African green MK cell cultures.

Results. Antigenic relationships among the HRVL agent, NCDV, EDIM virus, SA-11, and "O" agent. By CF each of the five reovirus-like agents reacted with its homologous hyperimmune antiserum to a titer which was similar, or in some instances identical, to that observed with each of the heterologous antisera (Table I). In addition, four children who had shed the reovirus-like agent and who had previously developed a serological response to the HRVL agent and NCDV not only again developed a seroresponse to these two viruses but also responded to the SA-11 and "O" viruses; also, three of the four developed seroresponses with the EDIM antigen (Table I). Thus, the five reovirus-like agents could not be differentiated by CF studies with hyperimmune animal or convalescent human sera. However, in tests with paired acute or cord and convalescent sera from two calves which developed illness following challenge with NCDV (8), the NCDV and the "O" agent each reacted with the convalescent serum of one of the calves to a titer of 1:32 whereas the other three reovirus-like agents failed to react with this serum; in addition, the NCDV and the "O" agent each reacted to a titer of 1:16 and the EDIM virus to a titer of 1:8 with the other calf's convalescent serum whereas the HRVL agent and SA-11 again failed to react. Antibody was not detected in the calves' preillness sera by any of the five antigens (<1:20 and <1:8 in such sera from the first and second calves, respectively).

Electron micrographs of the CF antigens used in Table I for the HRVL agent, NCDV, EDIM virus, SA-11, and the "O" agent are shown in Fig. 1. The morphologic similarity of the five reovirus-like CF antigens is striking (6, 10-13).

Serological tests with the five reovirus-like agents and paired acute and convalescent human sera. Since seroresponses were observed with the SA-11, "O," and EDIM viruses in tests with human paired sera, we attempted to compare the efficiency of these agents with that of the HRVL agent and the NCDV for detecting seroresponses in paired sera from infants and young children who had developed diarrhea and also shed the

TABLE I. Antigenic Relationships among the HRVL Agent, NCDV, EDIM Virus, SA-11, and "O" Agent with Hyperimmune Animal and Convalescent Human Sera by Complement Fixation[a]

		Reciprocal of serum antibody titer to indicated virus[d] (source of serum)					Reciprocal of child's acute/convalescent serum			
Virus	Source of CF antigen[b, c]	HRVL (guinea pig)	NCDV (guinea pig)	EDIM (mice)	SA-11 (guinea pig)	"O" (guinea pig)	Child 1	Child 2	Child 3	Child 4
HRVL	Stool filtrate (Da1A)	640	1024	1280	512	2048	<4/128	<4/64	<4/128	<4/128
NCDV	BEK cell cultures	1280	2048	1280	2048	8192	<4/32	<4/16	<4/32	<4/32
EDIM	Intestines of suckling mice	640	512	320	1024	4096	<4/32	<4/<4	<4/32	4/32
SA-11	African green monkey kidney cell cultures	1280	2048	2560	2048	8192	<4/32	<4/8	<4/16	<4/16
"O"	African green monkey kidney cell cultures	1280	2048	640	2048	4096	<4/32	<4/8	<4/32	<4/16

[a] All results in table obtained in same test.

[b] Titer of each antigen (0.025 ml) as calculated in simultaneous (straight line) titration in this test: HRVL agent, 2 units (tested with two of the convalescent human sera shown); NCDV, 1–2 units (tested with two convalescent NCDV calf sera); EDIM virus, 64 units; SA-11, 16 units; "O" agent, 32 units. EDIM, SA-11, and "O" viruses tested with the homologous hyperimmune animal serum shown. In a subsequent titration which included the same sera plus the hyperimmune animal sera for the HRVL agent and NCDV shown, the titers of the five viruses were as follows: HRVL agent, 2 units with convalescent human sera, 4 units with homologous huperimmune animal serum; NCDV, 8 units with convalescent calf sera and 64 units with hyperimmune animal serum; EDIM virus, 64 units; SA-11, 16 units; and "O" agent, 32 units.

[c] Control antigens described in Materials and methods were employed for each viral antigen and tested against each serum. Antibody was not detected in tests with pre- and postimmunization animal sera; in tests with human paired sera, antibody was not detected with control calf, control murine, and control simian antigens, while with control human stool filtrate antigen, antibody (1:8–1:32) was detected but without significant seroresponses.

[d] Antibody was not detected in any of the preimmunization sera in tests with all five reovirus-like antigens.

HRVL agent. As seen in Table II, of the 35 patients whose paired sera were studied, 26 (74%) developed serological evidence of infection with the HRVL agent, 15 (43%) with NCDV, 18 (51%) with EDIM virus, 20 (57%) with SA-11, and 25 (71%) with the "O" agent. The HRVL agent was significantly more efficient than the NCDV (χ^2 = 7.1, $p < 0.01$) and the EDIM virus (χ^2 = 3.9, $p < 0.05$) but not significantly more efficient than the SA-11 and "O" viruses for detecting a seroresponse. The NCDV was significantly less efficient than the "O" agent (χ^2 = 5.8, $p < 0.02$) but not significantly less efficient than the SA-11 and EDIM viruses. The table also shows that 31 (89.6%) of the 35 patients had a seroresponse to the HRVL and/or "O" agents and that concordance in the detection of the presence or absence of a seroresponse was found in 24 (69%) of the 35 paired sera tested with the HRVL and "O" agents. The geometric mean titers achieved in convalescent sera of seroresponders was significantly higher ($p < 0.001$, t test, two tail) with the HRVL agent than with any of the other four reovirus-like agents (Table II).

Relationship of SA-11, "O," and EDIM viruses to reovirus types 1, 2, and 3 and 20 orbiviruses by CF technique. In further CF tests, the SA-11, "O," and EDIM viruses did not react with a 1:4 dilution of a polyvalent reovirus guinea pig antiserum which reacted to a 1:32 dilution with reovirus types 1 and 2 and to a 1:64 dilution with reovirus type 3. In addition, reovirus types 1, 2, and 3 did not react by the CF technique with 1:8 dilutions of hyperimmune guinea pig sera to SA-11 and "O" viruses or with a 1:10 dilution of hyperimmune sera from mice immunized with EDIM virus. These hyperimmune sera had homologous titers of 1:1024, 1:2048, and 1:320 to the SA-11, "O," and EDIM viruses, respectively. The lack of a relationship of the SA-11 and "O" viruses with the three reovirus types, and the EDIM virus with reovirus type 3, is consistent with previous studies by other techniques (3, 14). The SA-11, "O," and EDIM viruses as well as reovirus types 1, 2, and 3 did not react with polyvalent ascitic fluids from immunized mice which contained CF antibody to 20 orbiviruses previously listed (1, 2).

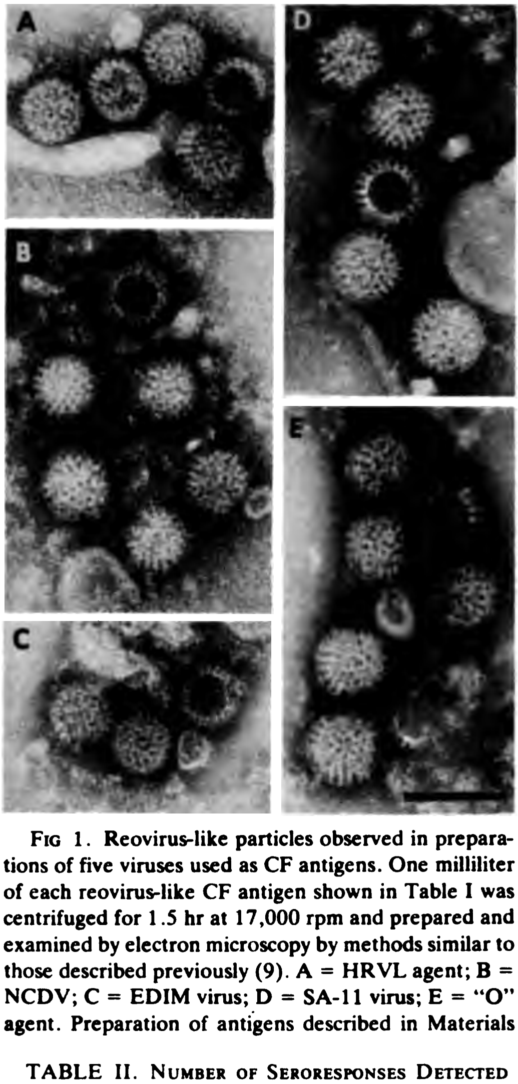

Fig 1. Reovirus-like particles observed in preparations of five viruses used as CF antigens. One milliliter of each reovirus-like CF antigen shown in Table I was centrifuged for 1.5 hr at 17,000 rpm and prepared and examined by electron microscopy by methods similar to those described previously (9). A = HRVL agent; B = NCDV; C = EDIM virus; D = SA-11 virus; E = "O" agent. Preparation of antigens described in Materials and methods. The bar = 100 nm and applies to A through E.

Discussion. We had previously shown that the cell culture-grown and concentrated NCDV could be utilized as a substitute CF antigen for detecting infection with the HRVL agent. The present studies demonstrate that three other related reovirus-like agents, the "O," SA-11, and EDIM viruses may also be used as substitute CF antigens. However, the "O" agent appears to be the most efficient of these four substitute CF antigens and, thus, it should be used preferentially when the HRVL agent is not available. The "O" agent was about as efficient as the HRVL agent and significantly more efficient than the NCDV for detecting seroresponses in patients shedding the HRVL agent. In addition, the "O" agent had another major advantage over the NCDV as a substitute CF antigen: It was easier to prepare since it did not have to be concentrated prior to use as CF antigen; unconcentrated cell culture harvests contained 16–64 units of CF antigen. The availability of the "O" agent as CF antigen should enable the efficient serodiagnosis of disease due to the HRVL agent. The greatest efficiency of detecting serological evidence of infection with the HRVL agent was achieved when sera were tested with both the HRVL and "O" agents as 31 (89%) of the 35 patients developed serological responses to either or both antigens.

TABLE II. Number of Seroresponses Detected to Five Reovirus-like Agents by CF in Paired Sera from 35 Diarrhea Patients Who Shed the HRVL Agent[a]

Antigen	Reciprocal of geometric mean titer in convalescent serum of seroresponders[b]	Patients who developed a seroresponse to indicated antigen (No. (%))	Patients who developed a seroresponse when results of CF tests with indicated pairs of antigens were combined (No.)				
			HRVL	NCDV	EDIM	SA-11	"O"
HRVL	28.0	26 (74%)	–	27	28	27	31
NCDV	15.3	15 (43%)	27	–	23	20	25
EDIM	10.5	18 (51%)	28	23	–	24	26
SA-11	12.1	20 (57%)	27	20	24	–	25
"O"	11.8	25 (71%)	31	25	26	25	–

[a] Fourfold or greater rise in CF antibody titer in paired sera.

[b] In acute phase serum of seroresponders, antibody was not detected (<1:4) in 23 of 26 with seroresponses to HRVL agent, in 14 of 15 with seroresponses to NCDV, in 17 of 18 with seroresponses to EDIM virus, in any of 20 with seroresponses to SA-11 virus, and in 24 of 25 with seroresponses to the "O" agent. With one exception, the maximum titer used in calculating the geometric titer (GMT) was 1:32 even though some convalescent sera had higher titers; 1:32 was used since all convalescent sera of seroresponders were titered at least up to this dilution. In the exception (with NCDV antigen) a single convalescent serum with a 1:64 titer was included since the acute serum of this pair had a 1:16 titer and to qualify for a seroresponse a fourfold or greater rise in antibody had to be observed (tabulating this serum as 1:32 resulted in a GMT of 1:14.6).

The finding of additional substitute CF antigens for the HRVL agent may also have important implications in the immunoprophylaxis against human infantile gastroenteritis caused by the HRVL agent (15, 16). Thus, efforts to find suitable strains of reovirus-like agents in various species should be pursued as it may be possible to immunize against the human disease utilizing an animal reovirus-like strain if one of these strains proves able to infect man without causing illness. Of course, attempts to find naturally occurring attenuated strains of the human agent or to develop such attenuated strains should be pursued as well as attempts to develop inactivated vaccines once the HRVL agent is grown efficiently in culture (17–20). Further studies will determine the course which should be pursued for immunoprophylaxis of this disease.

Summary. The human reovirus-like (HRVL) agent, Nebraska calf diarrhea virus (NCDV), epizootic diarrhea of infant mice (EDIM) virus, simian agent (SA)-11, and the "O" (offal) agent were found to be similar, if not identical, in reciprocal complement fixation (CF) tests employing hyperimmune animal sera. In addition, in CF tests with paired sera from 35 diarrhea patients who shed the HRVL agent, 74% developed serologic evidence of infection with the HRVL antigen, 43% with NCDV; 51% with EDIM virus, 57% with SA-11, and 71% with the "O" agent. Thus, in addition to the NCDV, which had previously been described as a suitable substitute CF antigen for the HRVL agent, the SA-11, "O," and EDIM viruses may also be utilized as substitute antigens for the HRVL agent. However, the "O" agent appears to be the most efficient of the four substitute CF antigens and thus should be used preferentially when the HRVL agent is not available. The "O" agent was about as efficient as the HRVL agent and significantly more efficient than the NCDV for detecting seroresponses. The greatest efficiency for detecting infection with the HRVL agent resulted when sera were tested with both the HRVL and "O" agents as 31 (89%) of the patients developed serologic evidence of infection with one or both antigens. The finding of additional substitute CF antigens for the HRVL agent may have implications in the immunoprophylaxis against human disease.

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Plasma Glucagon after Total Resection of the Pancreas in Man (39435)

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Although it has been reported that in dogs plasma glucagon not only does not disappear after pancreatectomy (1-3) but may actually increase if the animals receive no insulin treatment (4, 5), little is known about similar changes in man (6). Recently, we carried out serial measurements of plasma pancreatic glucagon and of glucagon-like immunoreactive materials during and after total resection of the pancreas in a patient with chronic calcified pancreatitis. The response of plasma pancreatic glucagon to arginine infusion was also examined.

Case report. Our patient was a 62-year-old man who did not drink alcoholic beverages and who underwent total resection of the pancreas, the duodenum, and the distal half of the stomach on July 1, 1974. Reconstruction of the alimentary tract was done as shown in Fig. 1. Severe pain and intermittent fever with leukocytosis had been present over a 5-year period. Conservative treatment had been ineffective and the patient had lost weight due to anorexia caused by frequent attacks of pain. Analgesic injections had been given three to five times daily for several months prior to surgery. Insulin administration had been discontinued following a decrease in blood glucose with loss of appetite and poor dietary intake. At the time of operation the patient weighed 40 kg.

The operative procedure was uneventful. The resected pancreas weighed 40 g and was completely calcified with multiple cystic formations in the ducts. Histological examination revealed fairly well preserved islets of Langerhans and markedly reduced acinar tissue.

Just before the final phase of pancreatectomy, infusion of regular insulin and glucose was started. The infusion of insulin was replaced by multiple subcutaneous injections of regular insulin from the 4th postoperative day on. The postoperative course of this patient has been uneventful up to the time of this writing, 16 months after the surgery. The preoperative complaints have disappeared and he has gained 3 kg.

Serial measurements of pancreatic glucagon and of glucagon-like immunoreactive materials were made during and after the operation until the 10th postoperative day.

Sampling. Peripheral venous blood was collected into heparinized syringes, plasma was separated immediately and stored at −20° until ths time of assay. Aprotinin[2] (1000 kIU/ml) was added to the plasma before refrigeration.

Assays. Plasma levels of glucagon (IRG) were measured by a single antibody radioimmunoassay (7) using an antiserum (AGS 30K), prepared in the laboratory of Dr. Roger Unger, Dallas, and found to be specific for pancreatic glucagon (8, 9). "True glucagon" (PG) values were corrected according to Weir's method (10), except that cellulose powder was used for the preparation of glucagon-free plasma (9). The assay system for PG in this study is capable of detecting changes of 25 pg/ml with 95% confidence. Fasting plasma levels of IRG and PG in healthy subjects were 223 ± 154 pg/ml (mean ± 1 SD, $n = 14$) and 37 ± 23 pg/ml (mean ± 1 SD, $n = 14$), respectively (9).

Plasma levels of glucagon-like immunoreactive materials (GLI) were also measured by a single antibody radioimmunoassay using AGS 10, prepared in the laboratory of Dr. P. P. Foà and kindly provided by Drs. K. Shima and T. Sugase. This antiserum cross-reacts with crude GLI prepared from the mucosa of the small intestine (11).

Results. As shown in Fig. 2, plasma IRG decreased progressively up to the end of the

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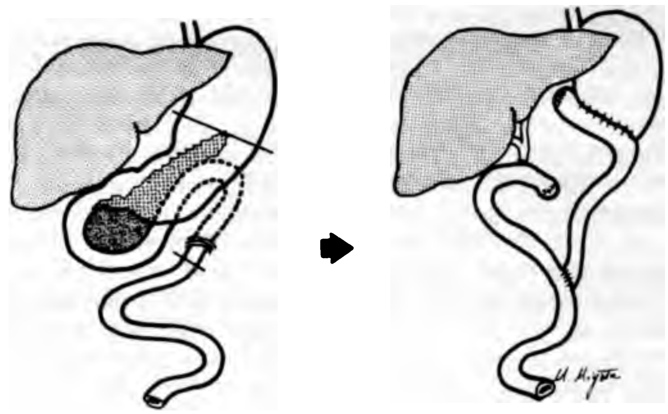

FIG. 1. Diagram of the operative procedure for total resection of the pancreas.

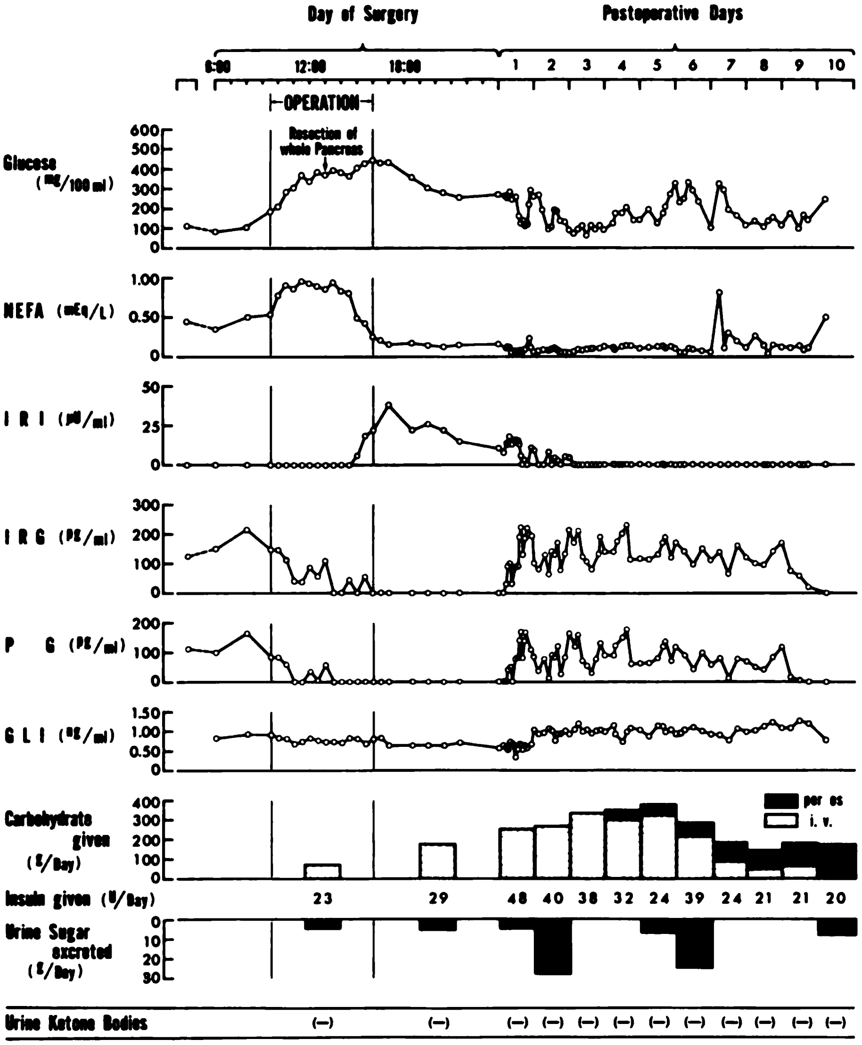

FIG. 2. Changes in plasma levels of glucose, nonesterified fatty acids (NEFA), insulin (IRI), glucagon (IRG), ue glucagon" (PG), and glucagon-like immunoreactive materials (GLI) during and after total resection of the creas.

operation and became undetectable until the first postoperative day. Plasma PG also decreased after the start of the operation and became undetectable within 30 min following the resection of the entire pancreas. On the first postoperative day, about 18 hr after total pancreatectomy, PG was again detectable in the blood and fluctuated considerably until the ninth postoperative day when it disappeared again.

Plasma levels of GLI gradually decreased after the start of the operation and during the second postoperative day, increasing abruptly thereafter, and remaining elevated until the ninth postoperative day. This abrupt increment of GLI coincided with the resumption of peristalsis after surgery.

As shown in Fig. 3, plasma IRG levels did not change in the blood following arginine infusion, even though the expected rise in growth hormone level was noted. PG did not appear in the blood during the infusion.

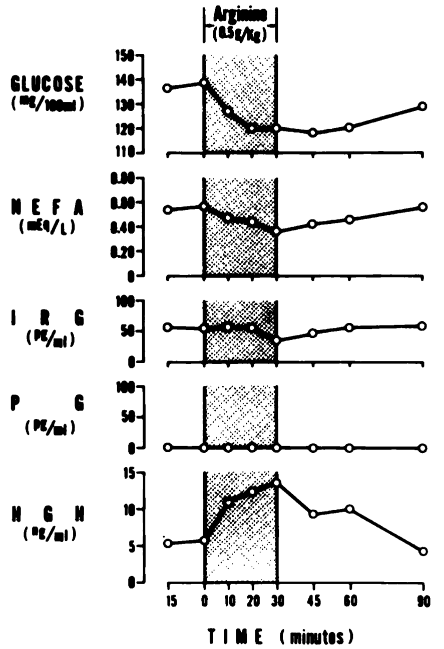

FIG. 3. Response of plasma glucose, nonesterified fatty acids (NEFA), glucagon (IRG), "true glucagon" (PG), and growth hormone (HGH) to arginine infusion during the fourteenth postoperative week. This examination was carried out after an overnight fast and 18 hr following the last injection of insulin. All values of "true glucagon" during the test were within the limits observed in the patient's plasma after adsorption on cellulose powder ("glucagon-free plasma").

Discussion. During surgery, independent observers confirmed that the pancreas had indeed been wholly resected and that accessory or ectopic pancreatic tissue could not be found in the abdomen. Thus, given a half-life of glucagon of about 4 min (12) there should have been no detectable PG in the blood of this patient after surgery. Nevertheless, variable amounts of PG were found between the first and ninth postoperative day. Since the fluctuation of PG did not parallel those of GLI and since AGS 30K cross-reacts minimally with GLI, the results strongly suggest that the immunoreactive material detected in the plasma after total pancreatectomy in this patient was not GLI. Similar findings have been reported in dogs (2, 3). Furthermore, in depancreatized dogs, the plasma level of PG increased with the withdrawal of insulin and was restored to almost normal values following the resumption of insulin treatment (4, 5). In our patient, plasma levels of glucose and nonesterified fatty acids (NEFA) were satisfactorily controlled by insulin, and ketone bodies were not detectable in the urine. Perhaps because of this, the maximum level of immunoreactive glucagon after total pancreatectomy was only twice the postoperative fasting level. On the other hand, plasma insulin (IRI) was not measurable except for the period when large doses of regular insulin were given shortly after pancreatectomy.

From the tenth postoperative day on, PG was not detectable in the blood of this patient and, during the fourteenth postoperative week, none appeared following arginine infusion. During the infusion, plasma glucose and NEFA decreased, probably due to the absence of glucagon. This lack of glucagon response is compatible with the observations of other investigators (6) and with our findings in another depancreatized patient. This 68-year-old male was admitted to the hospital, complaining of general weakness, 11 weeks after total resection of the pancreas for carcinoma. On admission (25 hr following the last injection of NPN insulin), the blood sugar level was 334 mg/100 ml, glucose (++) and ketone bodies (++) were detected in the urine. No detectable amounts of IRG and PG appeared in the plasma during arginine infusion. Thus, in depancreatized humans, ketosis does not appear to require the presence of measurable amounts of plasma glucagon. These observations on the absence of plasma glucagon response to arginine in patients after total pancreatectomy are compatible with the results obtained in well-insulinized depancreatized dogs (6), although not in untreated depancreatized animals (1, 2, 13).

Several possibilities may be considered to explain our findings: (i) release of PG from extrapancreatic tissue, (ii) appearance of unknown substances which cross-react with AGS specific for pancreatic glucagon, (iii) production of unknown metabolites which influence the radioimmunoassay. The most probable explanation appears to be the first one, since there is evidence that a pancreatic glucagon-like substance may be found in the mucosa of the gastric fundus and the duodenum (14), in the kidney (15, 16), and in the submaxillary gland (16–18). Indeed, the proximal half of the stomach was preserved in our patient. The facts that no plasma PG was detectable after the tenth postoperative day and that arginine infusion could not evoke a response may be due to insulin treatment, a procedure known to suppress glucagon secretion (5). It can be also speculated that, due to low levels of PG in the portal blood, insufficient amounts of this hormone may have escaped the liver to be detectable in the posthepatic blood.

The data described in this paper suggest that, also in man, "true" glucagon originates in organs other than the pancreas.

Summary. Serial measurements of plasma "true glucagon" (PG) and of glucagon-like immunoreactive materials (GLI) were carried out during and after total resection of the pancreas in a 62-year-old man with calcified pancreatitis. The postoperative course of this patient was uneventful and diabetes was well controlled. PG disappeared from the blood within 30 min after resection of the pancreas. In spite of the evidence that no pancreatic tissue was present in the abdomen, PG was detected again in the blood from 18 hr after total pancreatectomy until the ninth postoperative day. However, plasma PG did not rise following infusion of arginine during the fourteenth postoperative week. After an initial decrease, plasma GLI rose abruptly on the second postoperative day and remained elevated thereafter. The fluctuations of plasma PG and GLI were not parallel.

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Induction of Splenic Granulopoiesis *in Vitro*[1] (39436)

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The adult murine spleen is composed predominantly of lymphoid cells with some macrophages and granulocytic elements also present. Although the spleen accounts for only a small fraction of the blood cell production of the adult animal, it is an important hematopoietic organ containing both pluripotent stem cells (CFU-S) and committed granulopoietic progenitors capable of colony formation in soft-gel culture (CFU-C) (1-3). The concentration of CFU-C in the normal spleen is low relative to that in the bone marrow, and unlike the bone marrow CFU-C many splenic granulopoietic progenitors appear to be resting and not in active cycle (2, 4). Under conditions of increased demand for blood cells, the spleen of the adult, like that of the fetus, can become an actively hematopoietic organ. Although erythropoietin is clearly important in initiating splenic erythropoiesis, the signals for activation of splenic leukopoiesis are not clearly defined. In order to examine the characteristics of splenic granulopoiesis, we studied the generation of CFU-C and of mature granulocytes and mononuclear phagocytes in suspension cultures of spleen cells induced by colony-stimulating activity (CSA). In this system, CSA stimulation resulted in a marked proliferation of CFU-C and differentiation of granulopoietic progenitor cells.

Materials and methods. Young adult female DBA-2 mice (21-25 g) were used for all experiments. The spleen was removed aseptically and a single-cell suspension prepared by first mincing the tissue in culture medium, and then passing it through progressively smaller-bore needles until the cells passed easily through a No. 25 needle. Cell viability was greater than 99% as determined by trypan blue exclusion (0.4% dye over 4 min). McCoy's 5A medium containing 15% fetal calf serum and antibiotics was used throughout (5, 6).

Pregnant mouse uterus extract (PMU) was used as the source of colony-stimulating activity (CSA). The extract was prepared as described by Bradley *et al.* (7) and added to cultures at a concentration of 100 μl/ml. This concentration was previously shown to yield maximal colony formation in agar cultures of mouse bone marrow and spleen cells.

Spleen cells were cultured in liquid suspension with the Marbrook *in vitro* diffusion system previously described (6, 8). Cultures were initiated with 3×10^6 spleen cells in 1 ml of medium with or without the addition of 0.1 ml of PMU.

The suspension cultures were terminated at intervals up to 14 days for total viable cell counts (hemacytometer), and 200-cell differential counts were performed on Giemsa-stained cytocentrifuge preparations. Cultures were pulsed with 1 μCi of tritiated thymidine ([^{3}H]TdR) (sp act, 2 Ci/mmole) 60 min before harvest and autoradiographs prepared as previously described (6). A cell was considered labeled if its nucleus contained five or more grains above background. Labeling indices were expressed as the percentage of cells taking up the tracer and total labeled cells in culture calculated from the viable cell counts. At each period of harvest, a portion of the cultured cells was transferred to agar culture in order to assay the number of CFU-C present. Occasionally, there was spontaneous blastic transformation of lymphocytes *in vitro*. These cultures were discarded.

Agar cultures were prepared by the method of Pike and Robinson (5, 9). One-tenth milliliter of PMU was incorporated into an underlayer of 0.5% agar, and the cultured spleen cells were dispersed in a 0.3% agar overlayer. The number of cells

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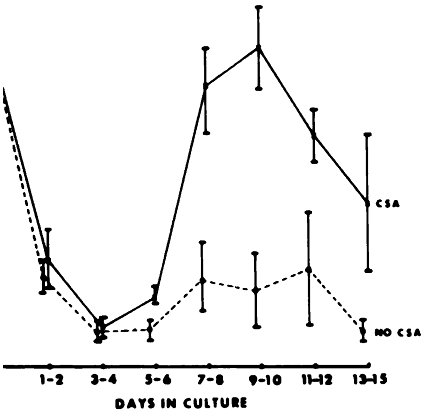
was adjusted so that the number of :s per plate ranged between 30 and uplicate agar cultures were incubated midified environment of 7.5% CO_2 in colonies containing 50 cells or more rated after 7–10 days of culture. The umber of CFU-C per liquid culture lculated by multiplying the plating ıcy in agar by the total viable cell

lts. The pattern of viable cell counts id culture is shown in Fig. 1. There apid fall in cell numbers until Day 4, ı control cultures and in those con- CSA. Thereafter, cell numbers in- l prominently in cultures to which ad been added, reaching a peak (four ontrol) at 9 to 10 days and remaining ıtially elevated above controls after 2 *:n vitro*. The increase in cell numbers observed in CSA-stimulated cultures was due to cellular proliferation as reflected by [^{3}H]TdR labeling studies (Table I). Prior to culture, there was only modest cellular DNA synthetic activity as indicated by the mean labeling index of 2%. In CSA-stimulated cultures the labeling index rose to a mean high of 47% at 5–6 days and total labeled cells increased by approximately 10-fold after 1 week of culture. Control unstimulated cultures showed considerably less increase in total labeled cells.



1. Viable cell counts in liquid cultures of :lls. Data represent means and standard errors experiments.

Differential cell counts revealed an increase in immature cells in CSA-stimulated cultures at 5–7 days, followed by the appearance of mature granulocytes and macrophages (Table II). At Day 5, about two-thirds of the cells were undifferentiated blast cells (Fig. 2). These cells subsequently differentiated appropriately to morphologically normal mature granulocytes and macrophages (Fig. 3). The mature cells were phagocytic for heat-killed *Candida albicans* and demonstrated cytochemical reactions typical of granulocytes and macrophages (10).

Data on the generation of CFU-C are given in Table III. Unstimulated cultures showed a modest increase in CFU-C with time. In CSA-stimulated cultures there was a mean 70-fold increase in granulopoietic progenitors capable of colony formation in agar. The rise in numbers of CFU-C preceded the appearance of morphologically identifiable blast cells which in turn preceded the increase in mature leukocytes (Fig. 4). The peak of the CFU-C curve roughly paralleled that of blast cell numbers. The increase in labeling index coincided with the increase in blast cells and CFU-C (Table I, Fig. 4).

ıBLE I. [^{3}H]Thymidine Labeling in Liquid Cultures of Spleen Cells (Seven Experiments)

	With CSA		Without CSA	
culture	Mean labeling index (%)	Total cells labeled (× 10^{-3} ± SE)	Mean labeling index (%)	Total cells labeled (× 10^{-3} ± SE)
0	2	60 ± 1	2	60 ± 1
1-2	5	50 ± 4	3	20 ± 6
3-4	30	120 ± 22	9	27 ± 9
5-6	47	300 ± 34	18	58 ± 14
7-8	25	650 ± 78	19	152 ± 24
9-10	10	290 ± 106	4	28 ± 7
1-12	5	111 ± 21	4	36 ± 20
3-16	3	42 ± 11	3	9 ± 3

TABLE II. Total Differential Cell Counts × 10^{-3} ± SE (Seven Experiments)

Days in culture	CSA	Immature cells	Mature granulocytes	Macrophages	Lymphoid cells
0		120	120	60	2700
5	+	448 ± 63	64 ± 9	38 ± 5	90 ± 13
	−	156 ± 41	14 ± 4	27 ± 7	143 ± 38
7-8	+	884 ± 136	854 ± 116	702 ± 108	160 ± 45
	−	304 ± 133	264 ± 99	152 ± 57	80 ± 35
9-10	+	510 ± 63	930 ± 114	1410 ± 174	150 ± 19
	−	91 ± 39	238 ± 102	322 ± 138	49 ± 21
11-12	+	88 ± 8	770 ± 84	1298 ± 142	44 ± 5
	−	9 ± 9	216 ± 120	648 ± 360	27 ± 15
13-15	+	120 ± 48	435 ± 174	945 ± 180	0
	−	0	26 ± 8	303 ± 92	0

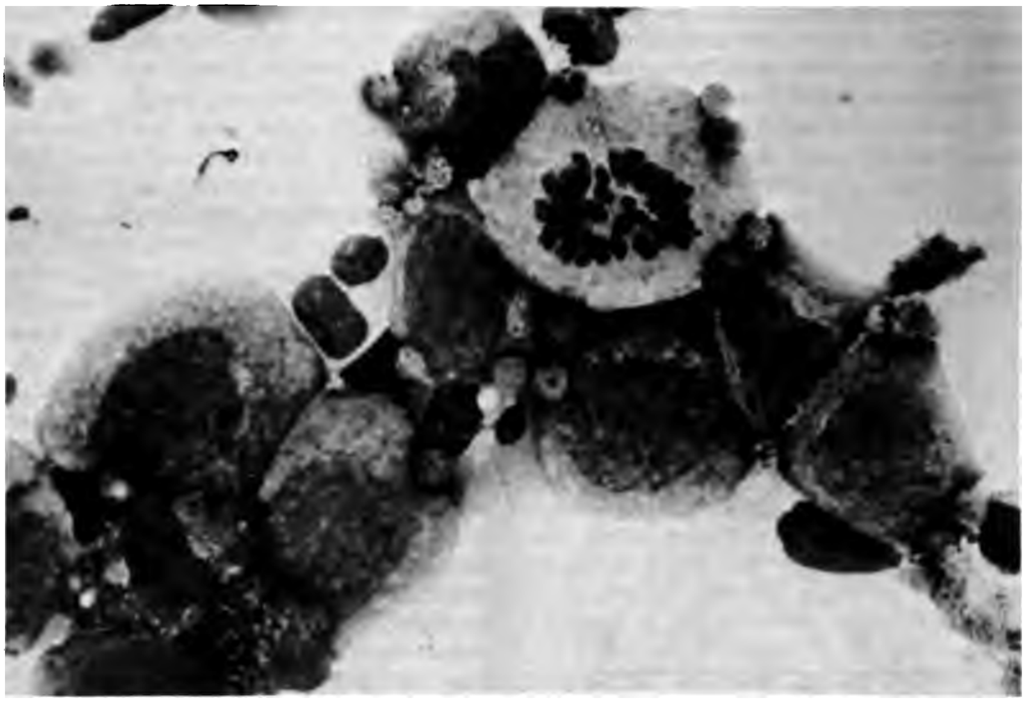


Fig. 2. Immature cells in culture at Day 5 (Giemsa stain).

Discussion. The adult spleen is generally regarded as predominantly a lymphoid organ composed of cells involved in immune reactions and as a reticuloendothelial organ whose mononuclear phagocytes are involved in the trapping and destruction of microorganisms and senescent blood cells. The spleen is also an important reservoir of hematopoietic activity. The hematopoietic potential of the spleen is usually expressed in fetal and perinatal life (1, 2) and during times of increased need for blood cells (hemorrhage or infection). In the adult mouse, the spleen contains pluripotent stem cells (CFU-S) (1, 3) as well as committed progenitors of erythroid cells (11) and granulocytes and monocytes. Committed granulopoietic stem cells are present in relatively low numbers and appear to be resting rather than in the proliferative cell cycle (2). In contrast, bone marrow contains a heterogeneous population of granulocytic precursors with concatenated proliferative and maturational activity.

The present studies demonstrate that splenic granulocytic and mononuclear phagocyte precursors can be induced by CSA *in vitro* to enter the active proliferative cycle, to generate additional committed stem cells (CFU-C), and to mature to nonproliferating

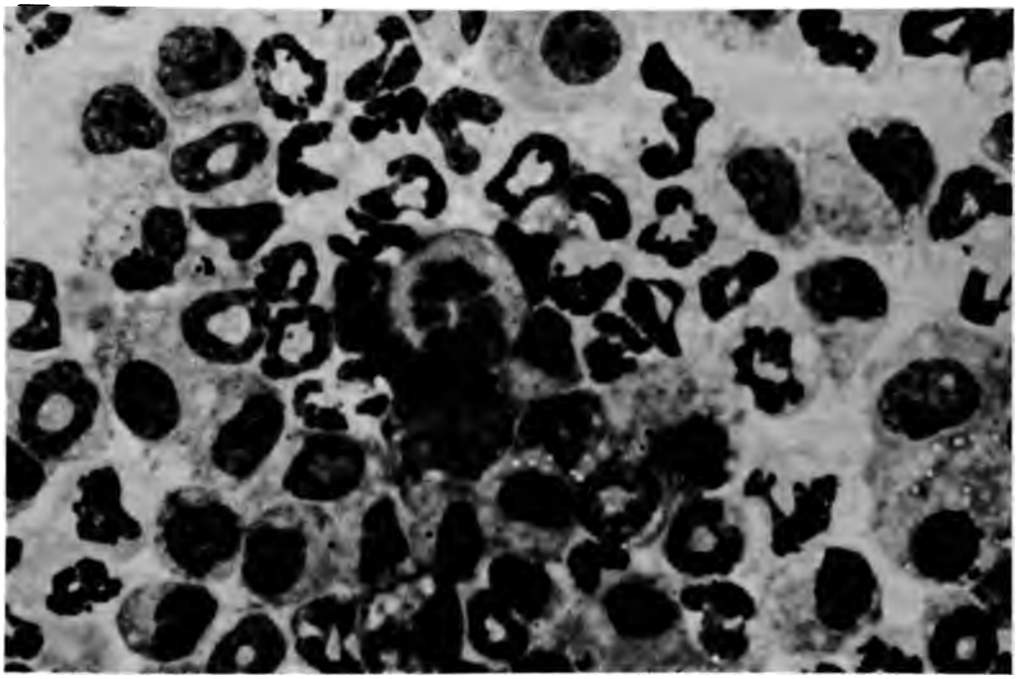


FIG. 3. Mature granulocytes and macrophages predominate in culture at Day 10 (Giemsa stain).

TABLE III. CFU-C IN LIQUID CULTURES OF NORMAL SPLEEN CELLS (FIVE EXPERIMENTS)

Time (days)	+CSA	−CSA
0	63 ± 13	63 ± 13
1-2	166 ± 32	70 ± 10
3-4	1272 ± 495	63 ± 2
5-6	3797 ± 1286	229 ± 68
7-10	4564 ± 707	913 ± 118
11-12	258 ± 117	197 ± 72

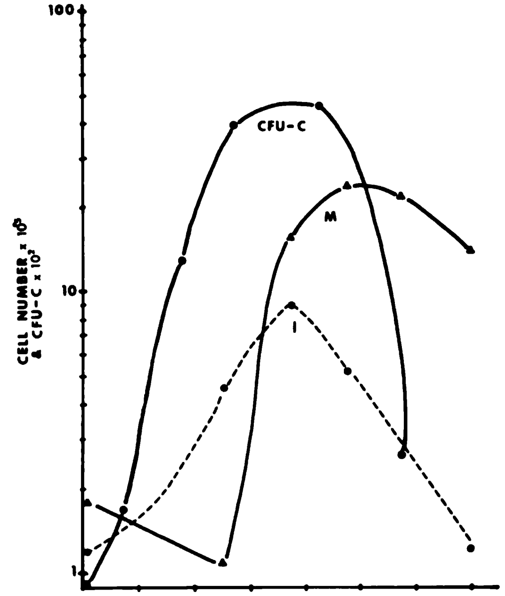


FIG. 4. Pattern of generation of CFU-C, immature cells (I) and mature (M) granulocytes and macrophages in CSA-stimulated cultures.

end cells. Cultures not containing CSA showed little proliferative activity and poor maintenance of cell viability. Under the influence of CSA, there was a rapid generation of CFU-C which resulted in a mean 70-fold increase over the number present at the initiation of culture. This increase in CFU-C was associated with a rise in [^{3}H]TdR labeling index from 2 to 47% after 5-6 days *in vitro*. Subsequently, viable cell counts increased and the labeling index fell with the appearance of mature granulocytes and macrophages. The very high labeling indices at Day 5 suggest that all or nearly all the proliferating cells are in cycle, representing a growth fraction near unity.

Various substances and environmental factors are known to stimulate DNA synthesis in the pluripotent stem cell (12, 13), and endotoxin specifically has been shown to cause a prominent increase in the number of such cells in the mouse spleen (14). The present observations are consistent with the hypothesis that CSA can be a physiologic inducer of committed granulopoietic stem cell proliferation in the spleen.

The orderly and uniform pattern of CFU-C generation, appearance of blast cells, and increased [^{3}H]TdR labeling index suggest that there is a synchronization of sequential proliferative and maturational activity. The liquid spleen cell culture system may thus provide a relatively simple means for studying the phenomena associated with stem cell activation and differentiation *in vitro*.

Summary. Murine spleen cells were cultured *in vitro* to study the induction of committed granulopoietic stem cell (CFU-C) proliferation and maturation. Marbrook-type diffusion cultures were established with and without the addition of colony-stimulating activity (CSA) and harvested at intervals up to 14 days for viable and differential cell counts, [^{3}H]TdR autoradiography, and quantitation of CFU-C by the agar plate method. Without CSA there was poor cell viability and little proliferative capacity. In CSA-stimulated cultures there was a prominent rise in viable cell counts and [^{3}H]TdR labeling indices rose from a mean of 2% at 0 time to 47% after 5 days *in vitro*. CFU-C increased by 70-fold in these cultures. Peak numbers of CFU-C, immature cells, and [^{3}H]TdR-labeled cells occurred at about 7 days. Thereafter, mature granulocytes and macrophages predominated in culture. Because the liquid spleen cell culture system begins in a resting state and undergoes a wave of proliferative activity in response to CSA, it can provide a useful model system for studying phenomena associated with stem cell activation and differentiation *in vitro*.

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Effect of Skin or Muscle Incisions or the Loss of Blood on Serum Leucogenenol Level[1] (39437)

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Leucogenenol, originally isolated from the metabolic products of *Penicillium gilmanii* (1), and demonstrated to be 2-(1,2-dihydroxy-3-methyl-5-oxocyclohexyl)-3,11-dihydroxy-11-(hydroxymethyl)-9-methyl-1-oxa-5-azaspiro [5.5] undeca-2,4-dien-7-one (2,3) is normally present and was isolated in concentrations of approximately 5 mg/kg (dry weight) from liver (4), thyroid, thymus, and gonads (5), and in concentrations of 0.06 mg/kg (human) to 0.6 mg/kg (bovine) from blood serum (6) where it is associated with a carrier protein (7).

Although leucogenenol has no significant effect on the rate of replication of or uptake of tritiated thymidine by lymphoblastoid cells in tissue culture, it does affect their oxygen uptake and carbon dioxide evolution and thus, their respiratory quotient (8–10). Under well-defined conditions, an optimum quantity of leucogenenol (1.9×10^{-4} μg/approx 10^5 cells) causes an easily measurable change in respiratory quotient (ΔRQ) of L5178Y cells of from 1.0 to 1.7. Greater or lesser than optimum quantities of leucogenenol have a significantly less effect on the ΔRQ (10) and the ΔRQ is related to the log of the reciprocal of the quantity of leucogenenol added to the medium by a gaussian type mathematical function. The latter observation made possible the development of an assay (6) in which the quantitative isolation of leucogenenol from a known volume of serum is carried to a stage where no compounds are present other than leucogenenol that affect the ΔRQ of L5178Y cells. The concentration of leucogenenol in the serum is then calculated from the dilution of a known volume of a solution of this partially isolated leucogenenol that causes a maximum ΔRQ of L5178Y cells. When known quantities of leucogenenol are added to samples of sera these quantities are accurately determined (within 5%) by the bioassay.

The concentration of leucogenenol in the serum varies with the species of animal (5,6) and in particular with abnormal conditions (11). A preliminary investigation of the levels of leucogenenol in the serum of patients with a variety of diseases showed that it is not significantly elevated in noninflammatory diseases but is significantly elevated in patients with diseases that are associated with tissue changes or destruction[2] such as systemic lupus erythematosus, rheumatoid arthritis, or subacute bacterial endocarditis.

Injection of leucogenenol into animals results in an increase in the rate of maturation of the myeloblasts and erythroblasts in their bone marrow (12–17), and addition of leucogenenol to a tissue culture of human peripheral lymphocytes induces blast formation (18). It is apparent that if leucogenenol also affects the rate of transformation and/or maturation of cells associated with tissue repair an elevation in serum leucogenenol might be expected in diseases associated with tissue destruction. It might also be predicted that artificially induced loss of, or injury to tissues of normal animals should result in elevated levels of leucogenenol in their sera.

We wish to report that skin or muscle incisions or the loss of appreciable quantities of blood result in the temporary elevation of the level of leucogenenol in the sera of rabbits and rats.

Materials and methods. Rabbits (New Zealand whites of 3 to 5 kg in weight) and rats (Sprague–Dawley, approximately 250 g in weight) were exposed to a light and dark cycle of 12 hr each, and allowed food (Pur-

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[2] M. B. Stevens, F. A. H. Rice, J. D. McCurdy, and C. G. Chen, unpublished data.

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ina Lab Rabbit or Rat Chow, as appropriate) and water *ad libitum*. Ether (Mallinckrodt, anesthesia grade) was used for anesthesia. Blood was drawn with sterile disposable syringes and the serum was obtained after allowing the blood to clot for approximately 1 hr at room temperature and overnight at 4°. The serum was removed with a disposable Pasteur pipet. The concentration of leucogenenol in the serum was determined as previously reported (5, 6).

Effect of a skin incision on the concentration of leucogenenol in the serum of rabbits. Two to three milliliters of blood was withdrawn from the marginal ear vein of each of 16 rabbits (8 males and 8 females). The serum was separated and assayed for its concentration of leucogenenol. At random intervals over a period of 3 weeks, blood was again withdrawn (altogether 42 samples) one or more times from the marginal ear vein of each rabbit, the serum was separated and assayed for its concentration of leucogenenol. Eight of the 16 rabbits (4 males and 4 females) then were selected at random, anesthesized, and 2 to 3 ml of blood was withdrawn from the marginal ear vein of each rabbit, the serum was separated and assayed for its leucogenenol concentration. One week later the eight rabbits were anesthesized again and an incision (approximately 10 cm) was made through the skin on the back of each rabbit midway between the neck and the tail. The incision was immediately closed with silk sutures and anesthesia discontinued. At intervals after closing the incision (1/4 to 1/2 hr, 1 hr, 3 hr, 5 hr, 24 hr, 3 days, and 6 days) 2 to 3 ml of blood was withdrawn from the marginal ear vein of three or more of the rabbits, the serum was separated and assayed for its concentration of leucogenenol.

Effect of the loss of blood on the concentration of leucogenenol in the serum of rabbits. Six rabbits (3 males and 3 females) were selected at random from the remaining eight rabbits and 25 to 30 ml of blood withdrawn from each rabbit by cardiac puncture under anesthesia. The serum was separated and assayed for its concentration of leucogenenol. Twenty-four hours and again 3 days later, approximately 5 ml of blood was obtained from each of the rabbits by cardiac puncture under anesthesia. The serum was separated and assayed for its concentration of leucogenenol.

Effect of skin and muscle incisions on the concentration of leucogenenol in the serum of rats. Twelve male and 12 female rats were exsanguinated (approximately 10 ml of blood) by cardiac puncture under anesthesia. The serum was separated from the blood of each rat and assayed for its concentration of leucogenenol. Twenty-eight male rats then were anesthesized and a right and left lateral rectus incision (approximately 2 cm) was made approximately 2 cm from the midline through the skin and muscles into the abdominal cavity of each animal. The muscle layers were closed immediately with gut sutures and the skin was closed with autoclips (Clay Adams, 9-mm No. B-2355/S, stainless steel). Anesthesia was discontinued then in all but seven rats which were exsanguinated immediately by cardiac puncture and the serum was separated and assayed for its concentration of leucogenenol. Five hours, 24 hr, and 3 days, after closing the incisions, seven of the remaining 21 animals were exsanguinated under anesthesia and the serum separated from their blood and assayed for its concentration of leucogenenol.

Effect of loss of blood on the concentration of leucogenenol in the serum of rats. Blood (approximately 2.5 ml) was withdrawn by cardiac puncture under anesthesia from each of six male and six female rats. The serum was separated and assayed for its concentration of leucogenenol. Blood (approximately 2.5 ml) was withdrawn again by cardiac puncture under anesthesia from each of the male rats at the following intervals after the first withdrawal: 1 hr, 1 day, 3 days, and 6 days. Blood (approximately 2.5 ml) was withdrawn from each of the female rats at the following intervals after the first withdrawal: 1, 3, and 6 days. As before, the serum was separated from the blood of each rat and assayed for its concentration of leucogenenol.

Results and discussion. Table I shows the effect of making a skin incision on the concentration of leucogenenol in the serum of rabbits. Normal male and female rabbits show no significant differences in the con-

TABLE I. EFFECT OF MAKING A SKIN INCISION[a] ON THE CONCENTRATION OF LEUCOGENENOL IN THE SERUM OF RABBITS.

	Concentration of leucogenenol in the serum at intervals following a skin incision (μg/liter)							
Rabbit No.[b]	0	1/4 to 1/2 hr	1 hr	3 hr	5 hr	24 hr	3 days	6 days
Males								
1	55.6	54.4		106.1	60.5	101.0	132.3	50.4
2	55.0	58.8	82.5	73.9	47.6	98.8		52.8
3	59.5		50.7	63.5	63.5	67.5		62.5
4	48.4		23.7	30.2	99.3	214.7	73.6	39.4
Females								
5	38.8	62.5	52.5	99.3	64.1	122.6	68.9	31.4
6	39.9			56.8	28.9	107.8	97.0	26.1
7	65.3				70.5	348.4		53.7
8	58.9				33.6	84.3	62.1	58.9
Mean	52.7	58.2	52.3	71.6	58.5	143.2	75.4	46.9
±S.D.[c]	±9.5		±24	±28	±22	±94[d]	±15[d]	±13

[a] An approximately 10 cm incision was made on the back of each rabbit midway between the neck and the tail. The incision was closed immediately with sutures. For details see text.

[b] Serum obtained at random two to four times over a 3 week period from eight male and eight female rabbits showed a mean concentration of leucogenenol of 50.8 ± 14 μg/liter (42 determinations).

[c] Standard deviation.

[d] $p < 0.001$ for difference from animals before the incision was made.

centration of leucogenenol in their serum (48.4 to 59.5 and 38.8 to 65.3 μg/liter, respectively). Neither is there a significant difference between the concentration of leucogenenol in the blood of unanesthesized animals and animals anesthesized with ether (50.4 ± 14 and 52.7 ± 9.5 μg/liter, respectively). Although 1/4 to 1/2, 1, 3, and 5 hr after making the skin incision the average concentration of leucogenenol in the serum of the rabbit is increased, the variation between animals, as indicated by the standard deviation, makes it impossible to determine whether or not it is significant. However, 24 hr after making the skin incision there is an unquestionable elevation in the concentration of leucogenenol in the rabbit's serum (143.2 ± 94 μg/liter vs the normal 52.7 ± 9 μg/liter). The concentration of leucogenenol in the serum remains elevated for at least 3 days, but returns to a normal value in 6 days.

Table II shows the effect of making two incisions into the abdominal cavity of rats on the concentration of leucogenenol in their serum. No significant change is observed 5 hr after making the incisions. However, the concentration of leucogenenol in the serum is elevated in 24 hr, but decreases to normal values in 3 days.

TABLE II. EFFECT OF MAKING TWO INCISIONS[a] INTO THE ABDOMINAL CAVITY OF RATS[b] ON THE CONCENTRATION OF LEUCOGENENOL IN THEIR SERUM.

Concentration of leucogenenol in the serum at intervals after closing the incisions (μg/liter ± standard deviation[c])			
0	5 hr	1 day	3 days
34.9 ± 3[b]	32.1 ± 11	75.0 ± 27[d]	33.8 ± 8

[a] Two approximately 2.5 cm lateral rectus incisions were made approximately 2 cm from each side of the median line and immediately closed with gut sutures (muscle layers) and autoclips (skin). For details see text.

[b] Male rats were used. An additional 12 male and 12 female rats showed normal concentrations of leucogenenol in their serum of 36.9 ± 9 μg/liter.

[c] Calculated from results on seven animals.

[d] $p < 0.001$ for difference from normal animals.

Table III shows that the concentration of leucogenenol in the serum of both male and female rats and rabbits is elevated the day after the rat has lost approximately one-quarter or the rabbit approximately one-half of the blood in its circulation. A second loss of the same quantity of blood from the rat the day following the initial loss results in the maintenance of the increased level of leucogenenol for at least 2 days. A third loss of approximately the same quantity of blood causes a further mean increase in the concentration of leucogenenol in the rat's serum.

TABLE III. Effect of the loss of Blood on the Concentration of Leucogenenol in the Serum of Rabbits and Rats.

	Concentration of leucogenenol in the serum at intervals following initial or repeated loss of blood[a] (μg/liter ± standard deviation)				
Animal	0	1 hr	1 day	3 days	6 days
Rabbits[a]	50.8 ± 14		162.2 ± 66[d]	76 ± 14	
Rats[b] (male)	32.0 ± 3	46.5 ± 8	96.6 ± 25[c,d]	109.0 ± 51[c,d]	278.4 ± 146[d]
Rats[b] (female)	37.8 ± 9		86.2 ± 25[c,d]	70.6 ± 28[c,d]	250.0 ± 90[d]

[a] Approximately 25 ml of blood was withdrawn by cardiac puncture under ether anesthesia from each of three male and three female rabbits. One day and 3 days later approximately 2.5 ml of blood was obtained from each of the rabbits by cardiac puncture under ether anesthesia. The serum was separated from each sample of blood and assayed for its concentration of leucogenenol. For details see text.

[b] Approximately 2.5 ml of blood was withdrawn from each of six male and six female rats by cardiac puncture under ether anesthesia at each of the indicated time intervals. The serum was separated from each sample of blood and assayed for its concentration of leucogenenol.

[c] Approximately 2.5 ml of blood again withdrawn.

[d] p <0.001 for difference from normal animals.

So far, most studies of the biological activity of leucogenenol have been concerned with its effect on the rate of maturation and/or transformation of blood cells (12–18) or associated phenomena such as antibody formation (19–22). However, there is no reason to conclude that leucogenenol does not have activity towards other cells as well. Most steroid hormones have more than one target organ (23) and growth hormone is not only necessary for general body growth but also is necessary for normal antibody formation (24).

Certainly it is likely to be more than a mere coincidence that damage done to tissues by skin or muscle incisions, ionizing radiation (11), and many inflammatory diseases[2] results in the elevation of the serum leucogenenol level. Fumarola and coworkers have demonstrated that leucogenenol induces a transformation of fibroblasts cultivated *in vitro* (25) and increases the rate at which colloidal carbon is cleared from the circulation of rabbits and rats (26). At least one should consider the possibility that an increased level of serum leucogenenol is a normal physiological response to many types of tissue damage, and that this elevation induces a more rapid formation of appropriate cells for repair and the clearance of debris.

Summary. It has been found that damage to a tissue of a rabbit or a rat, such as results from a skin incision or an incision through the skin and muscles into the abdominal cavity, is followed 24 hr later by a significant increase in the concentration of leucogenenol in the animal's serum. Likewise, loss of approximately one-quarter to one-half of the blood in the circulation of rabbits or rats causes an increase 24 hr later in the animals' serum leucogenenol concentration.

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Stability of Rauscher Leukemia Virus under Certain Laboratory Conditions (39438)

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Type C viruses are commonly encountered laboratory agents. Several type C viruses, including known oncogenic strains, replicate efficiently in human cells, representing biohazards of unknown potential. Virus mutants, conditionally or nonconditionally defective in replication, and viral recombinants, created deliberately or inadvertantly, are also potentially biohazardous (1). The National Cancer Institute (NCI) has formulated guidelines for the laboratory handling of biohazardous agents (2), but relatively little is known about the survival of type C viruses under laboratory encountered conditions. In this report we describe survival studies on Rauscher leukemia virus (RLV) (3) which may assist the further development of safe laboratory handling procedures.

Materials and methods. Biological laminar cabinet. All cell culture and virus procedures were performed in a 4-ft laminar flow biological safety cabinet class II (BioQuest) with an average vertical air flow velocity of 48 ft/min. Thermal temperatures of the cabinet work surface were initially ambient (25°), gradually increased to 28-29° after 3 hr of operation, and then remained steady. A new ultraviolet (uv) lamp (GE-G36T6) was installed into the provided cabinet fixture and activated 24 hr prior to use. The dose rate of the uv lamp was 500 $\mu W/cm^2$ at the work surface distance of 54 cm.

Virus. RLV stock was obtained from a chronically infected JLS-V9 subline (4) obtained from Dr. G. Shibley. The cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and antibiotics. Supernatant fluids were harvested at 24-hr intervals, clarified by centrifugation (4°, 12,000g, 10 min). Pooled samples were millipore filtered (0.45 μm), aliquoted, and stored at −70°. The virus was quick-thawed prior to use.

Virus assay. RLV was assayed by a direct focus assay. A1–2 cells are a clone of sarcoma virus positive, helper virus negative (S+H−)-transformed BALB/c peritoneal (BP) cells (5). Superinfection with B- or NB-tropic viruses results in the rapid development of foci of morphologically altered cells, similar to the S+L-assay of Bassin, *et al.* (6). Indicator cells were plated at a density of 1×10^5/60 mm plate and duplicate plates infected (1 hr, 37°) with 0.3–0.5 ml of virus dilution 24 hr later. Infected plates were fed with 7 ml of growth medium (Eagle's minimal medium supplemented with 10% heat-inactivated FBS and antibiotics). Foci were enumerated 5 days after infection.

Drying method and uv inactivation. Virus samples were dried by spreading (glass spreader 1 × 20 mm) undiluted virus (0.1 ml) over an area of 64 cm^2 on 150-mm integrid plastic plates (Falcon) and exposed to the air flow of the safety cabinet. Since preliminary experiments indicated that complete drying was achieved in 8–10 min, virus survival studies were performed on samples allowed to dry for 10 min. At various times after drying, 0.5–1.0 ml of cold medium was added, and the virus reconstituted by scrubbing with the glass spreader. This process was repeated, and the two samples were pooled and assayed immediately.

Ultraviolet inactivation was determined by exposing plates of dried virus immediately after drying to the uv lamp installed in the cabinet at a distance of 54 cm, and the virus reconstituted as described above.

Polymerase assay. RNA-dependent DNA polymerase (RDDP) activity was determined by a previously published method (7). Virus fluids were concentrated (4°, 100,000g, 1 hr) in a Beckman SW41 rotor and assayed for RDDP activity using the synthetic template $(dt)_{12-18} \cdot (rA)_n$ and results are expressed as Δ-pmoles of $[^3H]$dTMP product/ml of unconcentrated virus after 60 min of incubation at 37° (1 pmol = 19,422 cpm).

Results. The results presented represent

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the mean values of two to three experiments, and unless otherwise stated, were obtained using a single serum lot.

The thermal stability of RLV was tested at refrigeration (4°), ambient (25°), and incubator (36°) temperatures, and the data presented in Figs. 1 and 2 and Table I. Infectivity and RDDP activity were most stable at 4° and least stable at 36° (Table I). At all temperatures tested, RDDP activity was more stable than infectivity. This is demonstrated in Fig. 2 by the decrease with time of the number of FFU/pmol of RDDP activity.

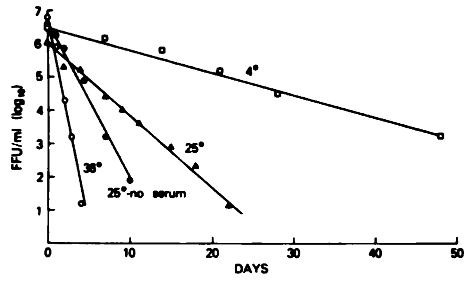

FIG. 1. Thermal stability of RLV. See Table I. Fetal bovine serum was omitted from the diluent of the specimen incubated at 25° and labeled "no serum."

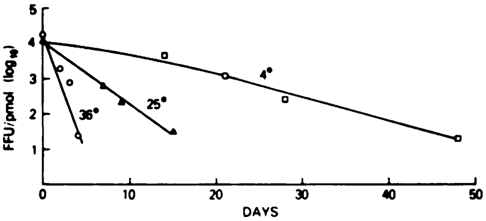

FIG. 2. Differential survival of RLV infectivity and RDDP activity. See Table I. The ratio of virus infectivity (FFU) to RDDP activity (pmol) is plotted as a function of time.

TABLE I. SURVIVAL OF RLV INFECTIVITY AND RDDP ACTIVITY.[1]

	Half-life	
Incubation temperature	Focus forming activity	RDDP activity
4	7 days	25 days
25	36 hr	10 days
36	7 hr	13 hr

[1] Stock RLV was diluted in growth medium and incubated in a refrigerator (4°) or water jacketed incubators (25° and 36°). Aliquots were periodically removed and tested for infectivity and RDDP activity. The figures represent mean values of two to three experiments.

The infectivity of virus suspended in serum-free medium decreased more rapidly than virus suspended in medium supplemented with 10% FBS. Serum lots varied considerably in their stabilizing effect, and the data presented were obtained with the optimal serum lots tested.

The survival of dried RLV was investigated (Fig. 3). A thin film of virus was allowed to dry in the safety cabinet for 10 min. Drying of the virus resulted in a tenfold reduction in titer. A further tenfold reduction in titer occurred during the first hour after drying. Subsequently, inactivation proceeded at a reduced rate, with an approximate half-life of 1 hr. When ethanol (70%) was sprayed onto the dried virus and permitted to evaporate prior to reconstitution, viable virus was not detected.

Figure 4 shows the inactivation with uv

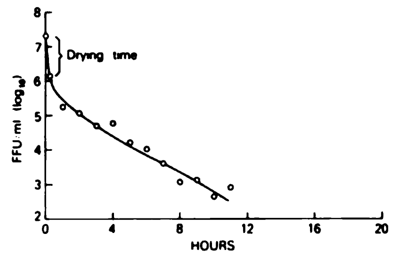

FIG. 3. Stability of air dried RLV in growth medium at 28-29° (Laminar cabinet temperature). Virus samples were spread on petri plates and air-dried in a safety cabinet under standard conditions, as described under material and methods. Samples on individual plates were reconstituted periodically and immediately assayed for surviving infectivity.

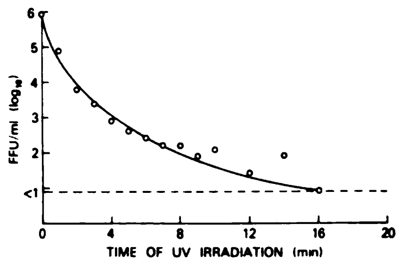

FIG. 4. UV inactivation of dried RLV. Virus samples were spread on petri dishes, air-dried in a safety cabinet (see materials and methods), and exposed to the uv light source installed in the cabinet (500 μW/cm²). Samples were periodically reconstituted and assayed immediately for surviving infectivity.

irradiation of air dried RLV using the light source installed in the safety cabinet. Sixteen minutes of irradiation inactivated over 5 logs of virus.

Discussion. Our results indicate that the half-lives of RLV at refrigeration, ambient, and incubator temperatures are 168, 36, and 7 hr, respectively. Virus suspended in FBS-supplemented medium survived longer than virus suspended in protein-free medium. These results are in general agreement with those of Levy *et al.* (8) for the survival of RLV oncogenicity, and of Smith (9) for the survival of Rous sarcoma virus infectivity. RDDP activity was more stable than focus-forming ability at all temperatures tested. The greater stability of RDDP activity should be considered whenever it is used as an indicator of infectivity.

After the first hour, air-dried virus had a half-life of approximately 1 hr. Exposure of dried virus to the uv source installed in the safety cabinet inactivated more than 5 logs in 16 min. Dried virus was inactivated also by spraying with 70% ethanol. The relative stability of dried virus and the known aerosol stability (10) and spread of RLV induced leukemia (11) increase the potential biohazard of type C viruses.

These studies indicate that RLV is relatively stable under a variety of laboratory encountered conditions, providing opportunities for accidental exposure of laboratory personnel, as well as inadvertant contamination of other virus stocks and cell cultures. Recommended laboratory procedures include the use of laminar flow safety cabinets, decontamination of the work surface and interior wall of the cabinet with ethanol, or other chemical agents, and exposure of the cabinet interior to uv light for at least 15 min between handling different cell cultures or viruses.

Summary. The stability of Rauscher leukemia virus (RLV) was investigated under certain laboratory conditions. The half life of the virus at 37° was 7 hr, and considerably longer at lower temperatures. RNA dependent DNA polymerase activity was more stable than infectivity at all temperatures. Air dried virus had a half life of approximately 1 hr, but was rapidly inactivated by uv light or 70% alcohol.

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Inhibition by Salmon Calcitonin (SCT) of Desoxycorticosterone Acetate (DOCA) Induced Hypertension in the Rat (39439)

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We have previously reported the capability of SCT to produce an acute diuresis and natriuresis in the saline loaded rat (1), as well as its ability to reduce the paw edema or inflammatory phase associated with the chronic disease state known as rat adjuvant arthritis (12). In addition, we have noted a significant decrease in the heart weight of normal rats given high doses of salmon calcitonin for 7 days (unpublished). We thus became interested in determining what effects SCT might have in a chronic hypertensive state such as that produced in rats by administration of DOCA in combination with a high salt intake.

Methods. Two separate experiments were performed in which female Sprague-Dawley rats with initial weights of 50–70 g were treated sc for 5 weeks with 5 or 25 mg/kg/dose of DOCA given b.i.d. with 1% NaCl in the drinking water. Synthetic salmon calcitonin (4000 MRC u/mg), purified porcine calcitonin (100 MRC u/mg), or synthetic human calcitonin (80 MRC u/mg) was administered sc at a dosage of 5 MRC u/kg/dose b.i.d. in a 16% gelatin vehicle concomitantly with DOCA but at a different injection site.[1] At the end of the treatment period direct measurements of aortic blood pressure were recorded in the unanesthetized state over at least a 5-min period following cannula insertion and pressure stabilization. Systolic, diastolic, mean blood pressure, and heart rate were recorded. The animals were then sacrificed and measurements made of heart and kidney weights. Appropriate control groups receiving distilled water or 1% NaCl to drink and injected with vehicle were included. Levels of statistical significance for group differences were determined using Student's *t* test.

Results. In Table I are shown the results of the blood pressure measurements in the two experiments after 5 weeks of treatment. Since systolic and diastolic pressures varied proportionally, only data on mean pressure are presented. Substitution of 1% NaCl for distilled drinking water had no effect on mean blood pressure. As may be observed in experiment 1, 5 mg/kg b.i.d. of DOCA given for 5 weeks had no effect on mean pressure, and SCT had no modifying effect on this dose of DOCA. However, 25 mg/kg b.i.d. of DOCA produced a highly significant increase in mean blood pressure in both experiments ($P < .001$) and the addition of 5 MRC u/kg b.i.d. of SCT to this dose of DOCA significantly reduced ($P < .001$) the hypertension in each experiment. It should be noted that although SCT produced a significant lessening of DOCA-induced hypertension, these animals were still significantly hypertensive compared to control animals receiving no DOCA. In experiment 2, groups of DOCA-treated rats were given purified porcine calcitonin or synthetic human calcitonin at equivalent hypocalcemic dosages as SCT with no significant reduction in the markedly elevated levels of blood pressure.

Measurements of heart rate (data not presented) show a statistically significant increase with 25 mg/kg b.i.d. of DOCA compared to the distilled water control in experiment 2 only. As this effect was not observed in both experiments, we do not consider this finding to be of major importance.

The effects on heart weight are shown in Table II. DOCA, at a dosage of 5 mg/kg b.i.d. had no effect on heart weight, while 25 mg/kg b.i.d. produced a highly significant increase in this parameter in both experiments. SCT decreased DOCA-induced cardiac hypertrophy in both experiments but this effect achieved statistical significance ($P < .02$) only in experiment 1. Por-

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[1] Calculated on a weight basis, the doses given were: SCT 1.25 μg/kg b.i.d.; porcine calcitonin 50.0 μg/kg b.i.d.; and human calcitonin 62.5 μg/kg b.i.d. All were manufactured by Armour Pharm. Co.

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TABLE I. EFFECT OF SCT ON MEAN BLOOD PRESSURE IN DOCA TREATED RATS.[a]

Groups No.	To drink	DOCA (mg/kg b.i.d.)	CT	Mean blood pressure (mm Hg)	
				Expt 1	Expt 2
1	H_2O	–	–	113 ± 8	120 ± 5
2	1% NaCl	–	–	113 ± 11	117 ± 8
3	1% NaCl	5	–	116 ± 11	–
4	1% NaCl	5	SCT	117 ± 8	–
5	1% NaCl	25	–	166 ± 18*	191 ± 21*
6	1% NaCl	25	SCT	131 ± 11**, ***	140 ± 10*, ***
7	1% NaCl	25	PCT	–	171 ± 18*
8	1% NaCl	25	HCT	–	186 ± 15*

[a] Values are mean ± 1 standard deviation; $N = 7$ rats/group.
* $P < .001$ vs groups 1 and 2.
** $P < .01$ vs groups 1 and 2.
*** $P < .001$ vs group 5.

TABLE II. EFFECT OF SCT ON HEART WEIGHT IN DOCA TREATED RATS.[a]

Group No.	To drink	DOCA (mg/Kg b.i.d.)	CT	Heart weight (mg%)	
				Expt 1	Expt 2
1	H_2O	–	–	320 ± 15	316 ± 7
2	1% NaCl	–	–	319 ± 16	314 ± 12
3	1% NaCl	5	–	333 ± 19	–
4	1% NaCl	5	SCT	327 ± 21	–
5	1% NaCl	25	–	450 ± 72**	394 ± 56**
6	1% NaCl	25	SCT	367 ± 29**, ***	359 ± 26**
7	1% NaCl	25	PCT	–	385 ± 28*
8	1% NaCl	25	HCT	–	455 ± 85*

[a] Values are mean ± 1 standard deviation; $N = 7$ rats/group.
* $P < .001$ vs groups 1 and 2.
** $P < .01$ vs groups 1 and 2.
*** $P < .02$ vs group 5.

cine and human calcitonin did not significantly modify DOCA-induced cardiac hypertrophy. Histologic evaluation of cardiac tissue has not yet been performed.

Measurements of kidney weight (data not presented) showed that 25 mg/kg DOCA b.i.d., but not 5 mg/kg DOCA b.i.d., resulted in a highly significant ($P < .001$) increase in kidney weight in both experiments. Treatment with SCT, PCT, or HCT did not significantly ($P > .05$) modify kidney hypertrophy.

Discussion. An antihypertensive effect of SCT in rat hypertension induced by DOCA administration and high salt intake has not, to our knowledge, been previously reported, although a significant lowering of blood pressure by SCT in the spontaneously hypertensive rat has been shown (9). The capability of natriuretic agents such as chlorothiazide and spironolactone to prevent or reduce DOCA-salt hypertension is well documented (3, 4).

In earlier studies we noted a significantly greater diuretic and natriuretic potency of SCT in the rat compared to PCT (1) or HCT (unpublished) using equivalent hypocalcemic doses. Similar observations concerning the relative effects of salmon and human calcitonin on renal electrolyte excretion in the rat have been reported by Williams *et al.* (5). These differences in diuretic and natriuretic activity appear to parallel the antihypertensive potencies now reported for the three hormones. It is tempting to speculate that this parallelism is the result of a cause and effect relationship; however, studies to examine patterns of urine electrolyte excretion in developing DOCA-salt hypertension have not been completed.

previously reported observations of
ility of SCT to reduce paw edema in
ljuvant arthritis (2) and our present
showing antihypertensive activity of
n DOCA-salt hypertension in the rat
mpatible with the possibility of a com-
underlying mechanism of action
gh a diuretic–natriuretic pathway in
two experimental diseases. However,
mechanism may be independent from
fect of SCT on the kidney since it has
reported that the antihypertensive ac-
f certain diuretics such as the benzo-
azines persists in patients who are re-
ry to their diuretic action due to their
rrupted use (3). As an alternative hy-
sis a mechanism of action of SCT can
nsidered based on the well-established
of calcitonin to produce compart-
l shifts in calcium (6). Such shifts can
capillary permeability and thus
modify a potentially edematous state
). Further studies are obviously re-
l to resolve the means by which calcitonin can modify the progression of these experimental disease states.

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Role of the Renin-Angiotensin System in Dogs with Perinephritis Hypertension[1]

(39440)

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The development of analogs of angiotensin II which effectively antagonize the vascular actions of angiotensin II has provided a means of evaluating the role of angiotensin II in maintaining the arterial pressure in various hypertensive models. The compound 1-sarcosine-8-alanine angiotensin II has been shown to be an effective competitive antagonist to angiotensin II in a variety of animal species, including the dog (1). The present study utilized this angiotensin antagonist to investigate the role of the renin-angiotensin system in maintaining the elevated arterial pressure in dogs with experimentally induced perinephritis hypertension.

Materials and methods. Nine mongrel, female dogs, ranging 18 to 23 kg in body weight, were used in these studies. All dogs were fed a standard diet which provided a daily intake of 65 mEq of sodium and 55 mEq of potassium. Water was available *ad libitum*. Arterial pressures were routinely measured three times each week by placing the conscious dog supine on a table and puncturing the femoral artery with a 22 gauge syringe needle attached to a pressure transducer; the arterial pressures were recorded on a Sanborn recorder. Only those dogs that consistently had control arterial pressures of less than 130 mm Hg were used in these studies.

The dogs were divided into two groups. The first group of five dogs was the normotensive control group. The second group of dogs consisted of those in which hypertension was produced by cellophane perinephritis. Each dog in the hypertensive group was anesthetized with sodium pentobarbital, 35 mg/kg body weight iv, and the left kidney was exposed by a flank incision with sterile surgical procedures. A sheet of cellophane (no. 215 PD, E. I. DuPont de Nemours and Co.) was placed around the kidney and was held in place by a silk suture tied loosely around the renal hilus. The incision was closed and the animal was allowed to recover. Two weeks later each dog was again anesthetized as before and the right kidney was removed through a flank incision by sterile surgical procedures. After recovery from surgery the routine measurements of arterial pressures were resumed.

After approximately 2 months, when the hypertension was well established, or after about 3 weeks of control arterial pressure measurements in the normotensive group, each dog was again anesthetized and a catheter of polyvinyl chloride (Fr No. 5) was inserted into the carotid artery toward the heart, for measurement of arterial pressure. The incision was closed, with the end of the catheter sutured just beneath the skin. Three to five days later the experiment was performed.

On the morning of the experiment the dog's rectal temperature was measured. A polyethylene catheter (PE 50) was inserted percutaneously into the saphenous vein for the administration of angiotensin II or the angiotensin II analog. The end of the arterial catheter was exposed and was connected by means of an extension tube to a pressure transducer for recording mean arterial blood pressure. Throughout the experiment the conscious dog was lying on the floor of the laboratory, tied loosely to a table leg, and appeared to be undisturbed by the procedures.

After the dog was completely prepared for the experiment and was lying calmly for 30 min, a 6 ml blood sample for plasma renin activity was obtained through the arterial catheter and was placed in a tube containing 0.06 ml of a 10% solution of ethylenediaminetetraacetate (EDTA); a 6 ml arterial

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[1] Supported by USPHS Grant No. HL 17366.

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sample was also collected for serum ıitrogen and plasma concentrations of m and potassium. The arterial blood ıre was recorded continuously while ıg of angiotensin II (1-asparagine-5- cine angiotensin II, Schwarz-Mann, in 1 ml of isotonic saline was injected ough the venous catheter. After the or response had subsided the arterial ıre was recorded continuously for two ls of 15 min each. The angiotensin II g 1-sarcosine-8-alanine angiotensin II ıen infused iv at a rate of 6 μg/min/kg dy weight for 45 min; the analog was ved in isotonic saline and was infused gh the saphenous catheter by means of iable-speed syringe pump at a flow of ıximately 0.5 ml/min. During infusion : angiotensin II analog the mean arte- lood pressure was recorded continu- . Additional blood samples for plasma activity were obtained after 15, 30, 5 min of analog infusion. Immediately stopping the analog infusion the pres- sponse to 2.5 μg of synthetic angioten- was again recorded. One hour after ing the infusion of the angiotensin II g the mean arterial blood pressure was recorded and a final blood sample for ıa renin activity was obtained.

: blood samples for plasma renin activ- ere centrifuged in a cold environment nd the plasma samples obtained were l frozen. The renin determinations in- d processing the plasma samples for generation of angiotensin I by the od of Schneider *et al.* (2). The angi- in concentration was determined by ay in the pentolinium-blocked, pento- al-anesthetized rat, with changes in al pressure as the response parameter; etic angiotensin II (Hypertensin-Ciba) was used as a standard. Plasma renin activity was expressed as nanograms of angiotensin generated by 3 hr of incubation per milliliter of plasma. All samples were assayed in triplicate and the average of the three determinations was the value recorded. Plasma concentrations of sodium and potassium were determined by flame photometry. Serum urea nitrogen determinations were performed by the clinical chemistry laboratory of the University Hospital.

Results. All four dogs in the hypertensive group developed a pronounced increase in arterial pressure. The mean arterial pressure in this group averaged 170 ± 11 (SEM) mm Hg as compared to the average mean arterial pressure of 100 ± 10 mm Hg for the five normotensive dogs. The arterial pressures in these two groups of dogs were significantly different ($P < 0.01$) when analyzed by Student's *t* test for group comparisons (3). The values for rectal temperature, serum urea nitrogen, and plasma concentrations of sodium and potassium, which are summarized in Table I, were the same in these two groups of animals.

The effect of the angiotensin II analog on mean arterial pressure in the normotensive dogs is summarized in Fig. 1. Infusion of the angiotensin antagonist in these dogs resulted in a slight initial increase in arterial pressure of 5–10 mm Hg, which returned to the preinfusion levels before 5 min of infusion. After the initial pressor response had subsided, no further changes in arterial pressure were observed during the 45 min of analog infusion. One hour after stopping the infusion of the analog the arterial pressure was still unchanged. Infusion of the angiotensin II antagonist into dogs with cellophane perinephritis hypertension resulted in a pronounced initial rise in arterial pressure

BLE I. VALUES FOR RECTAL TEMPERATURE, SERUM UREA NITROGEN, AND PLASMA CONCENTRATIONS OF SODIUM AND POTASSIUM IN NORMAL DOGS AND IN DOGS WITH PERINEPHRITIS HYPERTENSION.[a]

	Rectal Temp. (°F)	Serum urea nitrogen (mg%)	Plasma Na concentration (mEq/liter)	Plasma K concentration (mEq/liter)
l dogs (n = 5)	101.9 ± 0.3	14 ± 2	145.2 ± 0.7	4.5 ± 0.2
phritis hypertensive (*n* = 4)	101.5 ± 0.2	17 ± 3	143.0 ± 0.6	4.3 ± 0.1

alues are means ± SEM. There were no statistical differences between the two groups when compared by t's *t* test.

which did not subside nearly as rapidly as in the normotensive dogs (see Fig. 2). Comparing the arterial pressure after 15, 30, and 45 min of analog infusion with the arterial pressure during the control period by Student's *t* test for paired observations revealed no statistically significant differences at any of these time periods. The arterial pressure also was unaltered 60 min after stopping the infusion of the analog. In no instance did a fall in arterial pressure occur with angiotensin II analog infusion in the hypertensive dogs. Prior to infusion of the analog the arterial pressure was stable in both groups of dogs, as indicated by the averages for the mean arterial pressures during the two control periods. The iv injection of 2.5 μg of angiotensin II during the control period produced a rise in mean arterial pressure averaging 78 and 71 mm Hg for the control and hypertensive dogs, respectively; at the termination of the analog infusion this dose of angiotensin II did not result in any changes in arterial pressure.

The average values for plasma renin activity are given in Table II. During the control period the values for plasma renin activity were similar in both groups of dogs, and no changes in plasma renin activity occurred during infusion of the angiotensin analog in either group.

Discussion. In recent years several analogs of angiotensin II have been developed which will antagonize the effects of angiotensin II in biological systems. The ability of the compound 1-sarcosine-8-alanine angiotensin II to block the vascular action of angiotensin II was first reported by Pals *et al.* (4) who studied the effect of this compound on the rabbit aortic strip *in vitro* and *in vivo* in the rat. Studies on dogs by this laboratory (5) have shown that infusion of this angiotensin analog into two preparations with elevated levels of plasma renin activity and increased aldosterone secretion, i.e., sodium-depleted dogs and dogs with a constriction of the thoracic inferior vena cava, resulted in a fall in arterial pressure and a decrease in the adrenal secretion rate of aldosterone; these results indicated that

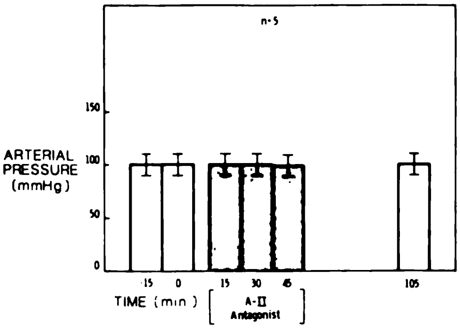

FIG. 1. Effect of iv infusion of 1-sarcosine-8-alanine angiotensin II, at 6 μg/min/kg of body weight, on arterial pressure in five normotensive dogs.

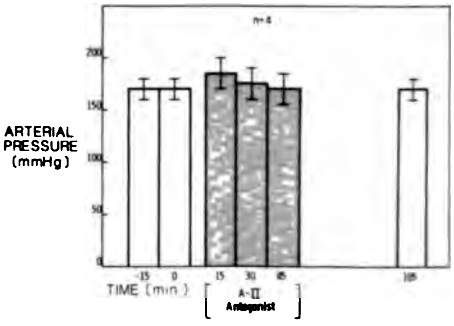

FIG. 2. Effect of iv infusion of 1-sarcosine-8-alanine angiotensin II, at 6 μg/min/kg of body weight, on arterial pressure in four dogs with cellophane perinephritis hypertension.

TABLE II. EFFECT OF THE INFUSION OF THE ANGIOTENSIN II ANALOG 1-SARCOSINE-8-ALANINE ANGIOTENSIN II ON PLASMA RENIN ACTIVITY IN NORMAL DOGS AND IN DOGS WITH PERINEPHRITIS HYPERTENSION.[a]

		Minutes of infusion of analog			
	Control	15 min	30 min	45 min	Recovery
Normal dogs (n = 5)	5.9 ± 0.8	6.8 ± 1.2	5.6 ± 1.3	6.4 ± 1.5	6.1 ± 1.0
Perinephritis hypertensive dogs (n = 4)	5.4 ± 1.0	5.5 ± 1.4	5.0 ± 1.4	5.5 ± 1.0	5.5 ± 1.3

[a] Values are means ± SEM. Renin values are nanograms of angiotensin generated during 3 hr incubation, per milliliter of plasma.

mpound blocked both the vascular
e steroidogenic effects of endoge-
produced angiotensin II in the dog.
mpound has been used to study the
f angiotensin II in maintaining the
d arterial pressure in dogs with ex-
ntal hypertension produced by renal
stenosis and contralateral nephrec-
Dogs prepared in this way that devel-
chronic hypertension with normal
f plasma renin activity did not show a
ion in arterial pressure during infu-
1-sarcosine-8-alanine angiotensin II
however, in those dogs that devel-
malignant form of hypertension with
d levels of plasma renin activity, the
n of this analog resulted in a reduc-
arterial pressure. Similar results have
eported by Sweet *et al.* (8) for dogs
nal artery stenosis and chronic hyper-
receiving another angiotensin antag-
1-sarcosine-8-isoleucine angiotensin

ough the production of experimental
hypertension by cellophane perine-
in animals was first reported by Page
9 (9) and this renal hypertensive
has been studied by many investiga-
he mechanisms whereby this proce-
sults in an elevated arterial pressure
unknown. Studies reported by Ferra-
l. (10) showed an increase in cardiac
early during the course of the devel-
t of cellophane-perinephritis hyper-
dogs; these workers postulated that
e in cardiac output resulted from an
ed venous return due to increased
tone. Mogil *et al.* (11) found plasma
activity to be normal or reduced in
ith chronic hypertension produced by
ane perinephritis, and concluded
e hypertension was not maintained by
ed activity of the renin–angiotensin
. The present study likewise found
l values for plasma renin activity in
ith perinephritis hypertension. Fur-
re, the infusion of the angiotensin II
nist 1-sarcosine-8-alanine angioten-
into these hypertensive dogs did not
e a lowering of the arterial pressure
of these hypertensive dogs. Similar
s have been reported by Bumpus *et*
) who infused the angiotensin antagonist 1-sarcosine-8-isoleucine angiotensin II into dogs with perinephritis hypertension. These findings provide strong evidence that the renin–angiotensin system is not involved in maintaining the elevated arterial pressure in dogs with chronic hypertension produced by cellophane perinephritis.

The slight pressor response of 5–10 mm Hg seen initially during the infusion of the angiotensin analog in the normotensive dogs has been reported previously by this laboratory (5) and has also been observed by other workers (7). Also, dogs with renal artery stenosis and hypertension similarly exhibit only a slight pressor response initially during the administration of this compound (6). Presumably this pressor response is mediated by catecholamines, since it is well established that angiotensin II will stimulate the release of adrenal medullary catecholamines (13–15), and an aromatic C-terminal amino acid is not necessary for this effect of angiotensin II (16). Also, Muñoz-Ramírez *et al.* (17) found that the initial pressor response to 1,8-substituted angiotensin II analogs in rats was attenuated by adrenalectomy or by alpha-adrenergic blocking agents. This leads to speculation that dogs with cellophane perinephritis hypertension may release more adrenal medullary catecholamines in response to angiotensin than do normal dogs or dogs with renal artery stenosis and hypertension, or that in dogs with cellophane perinephritis hypertension there may be a greater vascular reactivity to catecholamines.

Summary. Cellophane perinephritis hypertension was produced in four dogs, while five additional dogs served as normotensive controls. A competitive antagonist of angiotensin II, 1-sarcosine-8-alanine angiotensin II, was infused iv into these conscious dogs at a rate of 6 μg/min/kg of body weight for 45 min. Arterial pressure averaged 170 ± 11 (SEM) mm Hg in the dogs with perinephritic hypertension, and was not altered significantly during infusion of the angiotensin antagonist. In the normal dogs the arterial pressure averaged 100 ± 10 mm Hg and likewise, did not change during administration of the angiotensin analog. Plasma renin activity values were essentially the same in these two groups of dogs and did not change

during infusion of the angiotensin antagonist. These studies provide strong evidence that the renin–angiotensin system is not involved in maintaining the elevated arterial pressure in dogs with chronic hypertension produced by cellophane perinephritis.

The authors gratefully thank Dr. A. W. Castellion of Norwich Pharmacal Company for supplying the 1-sarcosine-8-alanine angiotensin II used in these studies. The cellophane used to produce perinephritis hypertension was supplied by E. I. DuPont de Nemours and Company. Mr. Charles Payne assisted in the surgical procedures and the assays for plasma renin activity were performed by Ms. Jane Green.

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GS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **152**, 565–567 (1976)

Direct Stimulation of Renin Release by Calcium[1] (39441)

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hough it is well known that calcium a vital role in both endocrine and exo- secretion (1), its role in the secretion nin is less clear. Thus, some authors st that calcium retards renin release), while others have provided evidence alcium enhances renin release (4, 5). ave examined the direct effect of cal- on renin release *in vitro*, independent ural or vascular control. Our results te that calcium can directly stimulate lease of renin.

thods. The technique we have adopted reviously been utilized to demonstrate lirect stimulant action of calcium on al medullary secretion, namely, rein- ction of calcium after a period of cal- depletion (6). Male Holtzman rats -300 g) maintained on regular diet killed by a blow to the head and both ys were then removed. Renal cortex eparated and the capsule was removed sing a razor blade. The cortex, thus ned, was cut into slices by hand, aver- 14 mg each. All of these procedures performed at room temperature. Nine were skewered on a long needle and ated at 37° in 2-ml volumes of oxygen- modified Krebs solution containing): NaCl, 140; KCl, 4.0; $CaCl_2$, 1.0; ose, 10; Tris buffer (pH 7.4), 10. a 30 min preincubation period the were transferred to successive tubes at intervals and aliquots of the medium assayed for renin released from the . The standard method of stimulation ted was to expose the tissues to cal- free conditions for 30–35 min and reintroduce calcium (0.5 m*M*) for 1 followed by the Ca-free medium again. n was assayed by radioimmunoassay of tensin I (7) generated from bovine serum substrate (8) at pH 7.0. The bovine serum substrate contained NaCl (150 m*M*), EDTA (10 m*M*), and phosphate buffer (pH 7.0, 50 m*M*). The final incubation mixture of renin and serum substrate contained 10 m*M* Cleland's reagent (DDT, dithiothreitol) and 3.3 m*M* EDTA. After incubation for 90 min at 37° the mixture was chilled on ice, diluted ninefold with 100 m*M* Tris buffer (pH 7.4) and assayed for angiotensin I (7). Angiotensin generation was proportional to renin concentration in the range utilized. Renin release is expressed as nanograms of angiotensin generated per hour of incubation with substrate per 100 mg tissue per minute of tissue incubation (ng/hr/100 mg/min).

Results and discussion. After 30 min incubation, the spontaneous release of renin reached a low and steady level, about 10–20 ng/hr/100 mg/min. If the kidney slices were pretreated with calcium-free medium, the reintroduction of calcium (0.5 or 6.0 m*M*) caused an immediate increase in renin release (Fig. 1). However, in the absence of calcium depletion (calcium maintained at 1.0 m*M*), addition of 0.5 or 6.0 m*M* calcium did not cause an increase in renin release (Fig. 1). To rule out secondary effects of calcium on Na^+ fluxes, experiments were done with Na-free medium (choline chloride replacing NaCl). Again, reintroduction of calcium caused a large increase in renin release (Fig. 2). Since the calcium-induced release of hormones from the posterior pituitary gland and from the adrenal medulla is dependent on a source of metabolic energy (9, 10), we have examined the effects of lowered temperature on the calcium-induced release of renin. When experiments were done at 20° instead of 37°, the release of renin in response to calcium was reduced (Fig. 3).

The present results parallel experiments on adrenal medullary secretion. It has been

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pported by grants from the Kansas Heart Asso- and the American Heart Association.

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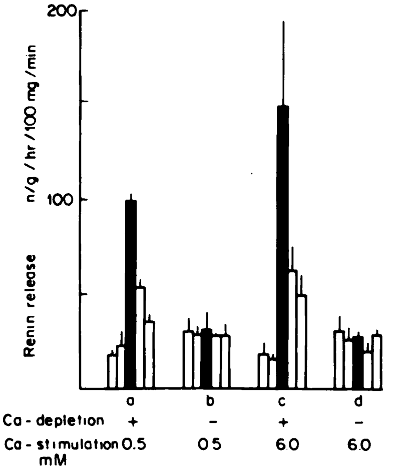

FIG. 1. Calcium-induced renin release. The vertical bars show renin released from four sets of rat kidney cortex slices (a–d). Slices were preincubated for three 10 min periods in Krebs solutions containing $CaCl_2$, 1.0 m*M* (b and d) or 0 (a and c). In the Ca-free experiments, EGTA, 1.0 m*M*, was present during the first 10 min period of preincubation. Renin released in five consecutive 1 min periods following the preincubation is shown in each experiment from left to right. In the third period (closed bars), calcium was added (0.5 m*M* in a and b; 6.0 m*M* in c and d). Renin release is expressed in ng angiotensin 1/hr substrate incubation/ 100 mg tissue/min tissue incubation. *N* = 3 for a–c and 5 for d. Each sample in each experiment was assayed in duplicates or triplicates. The thin line at the top represents the standard error.

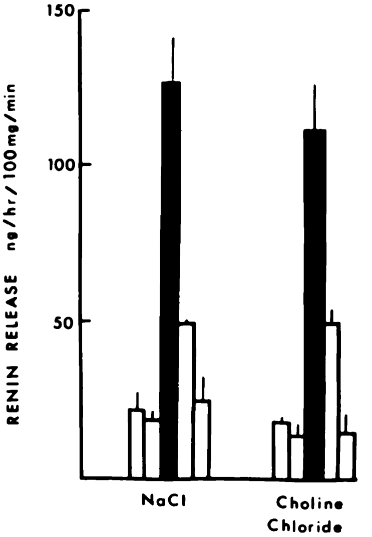

FIG. 2. Effect of NaCl replacement on calcium-induced renin release. The vertical bars show renin released from two sets of rat kidney cortex slices. Both sets of slices were preincubated for three 10 min periods in Ca-free Krebs solution, with 1.0 m*M* EGTA present during the first 10 min. In one set of slices, the NaCl in the Krebs solution was replaced by isotonic choline chloride throughout the experiment. Renin released in five consecutive 1 min periods following preincubation is shown from left to right in each experiment. In the third minute Ca (0.5 m*M*) was added. *N* = 3.

suggested that exposure of tissues to calcium-free medium increases their permeability to calcium so that subsequent treatment with calcium allows an enhanced calcium influx which then stimulates secretion (6). This may also be true for the juxtaglomerular cells and suggests that calcium can

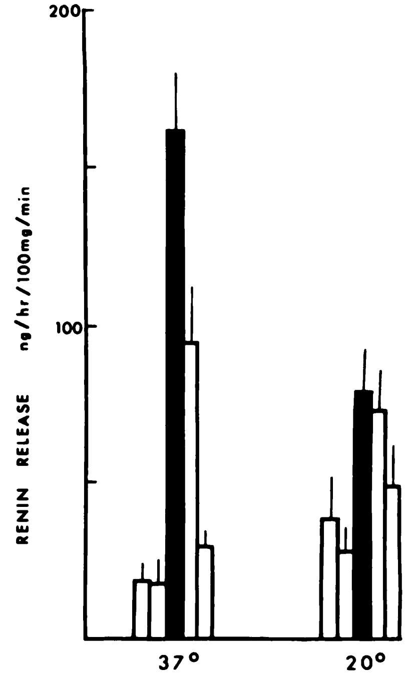

FIG. 3. Effect of temperature on calcium-induced renin release. The vertical bars show renin released from two sets of rat kidney cortex slices. Slices were preincubated for three 10 min periods in Ca-free Krebs solution, with 1.0 m*M* EGTA present during the first 10 min. Renin released in five consecutive 1 min period following preincubation is shown from left to right in each experiment. Calcium (0.5 m*M*) was added during the third min. One experiment was done at 37° and the other one at 20°. *N* = 4.

ly stimulate renin secretion. The pres-
xperiments, which were designed to
ıate the influence of neural, vascular,
sodium-dependent mechanisms on
secretion, show that the cellular basis
ıin release may be similar to that oper-
in catecholamine secretion. Further
:s will be needed to determine if there
ifferences from the more established
:ory systems.

nmary. Calcium directly stimulates
release from rat kidney slices previ-
treated with calcium-free medium.
,timulant effect of calcium (0.5 or 6.0
is not seen without the period of cal-
depletion. The stimulant effect of cal-
is still present in sodium-free medium
; reduced when the incubation is per-
:d at 20° instead of 37°. The results
st that the underlying mechanism of
release may be comparable to that of
ıolamine release, involving calcium-
ıdent and energy-dependent steps.

We acknowledge the valuable technical assistance of Mr. John Gillespie and Mrs. Roselle Poisner.

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Impaired Hepatic Heme Synthesis in the Phenobarbital-Stimulated Selenium-Deficient Rat (39442)

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In 1957 Schwarz and Foltz (1) described an association between dietary selenium deficiency and hepatic necrosis. However, although the dietary requirement for selenium is established, its possible role in the maintenance of normal hepatic function remains speculative. Diplock *et al.* (2, 3) have recently suggested that selenium may be incorporated into a nonheme iron containing microsomal protein active in the NADPH-dependent electron transfer chain. It is conceivable, therefore, that the integrity of the hepatic microsomal mixed-function oxidase system may in part be dependent on an adequate dietary supply of selenium.

We have recently described an impaired inducibility by phenobarbital of cytochrome P_{450} and cytochrome b_5 in the selenium deficient rat (4). The normal inducibility of the flavoprotein component of the mixed function oxidase system, NADPH cytochrome *c* reductase, in these animals suggested that the impaired response in these selenium deficient rats may be limited to the heme-containing microsomal proteins. Preliminary observations (4) indicated that the apoprotein component of cytochrome P_{450} was induced by phenobarbital in the selenium deficient rats. The present study is a continuing investigation of the possible discoordinated synthesis of heme and apoprotein in the selenium deficient phenobarbital-stimulated rat.

Materials and methods. Male Holtzman rats weighing 60–90 g were placed on a selenium deficient torula yeast diet (5) and water *ad lib*. Two dietary groups were formed by the addition of supplemental selenium, 0.5 mg/kg as Na_2SeO_3, to the diet of one group of animals. Thus, a "selenium replete" and "selenium deficient" group was formed; the animals were maintained on this diet for 3 months, at which stage animals in both groups weighed 300–350 g.

Four animals from each group were then subdivided into two further groups. Within each dietary group two animals received a daily intraperitoneal injection of phenobarbital (80 mg/kg in 0.9 NaCl) for 5 days, the remaining two animals receiving only the vehicle. The rats were then fasted overnight and each animal was given 10 μCi of 5-δ-amino-[^{14}C]levulinic acid (Amersham Radiochemical Centre, 14.4 mCi/mmole) by intraperitoneal injection. The animals were killed by exsanguination 2 hr later. The livers were removed and homogenized in 4 vol of cold 0.25 *M* sucrose, 0.01 *M* Tris-HCl pH 7.4 in a Potter–Elvejham homogenizer with Teflon pestle at 600 rpm. Subcellular fractions were separated by differential centrifugation by the method of Novikoff and Heus (6), and the microsomal subfraction was resuspended to 25 ml in 0.05 *M* Tris-HCl pH 7.4.

Aliquots of homogenate and the microsomal subfractions were used for the extraction of and determination of heme. Lipids were first extracted as described by Druyan and Kelly (7). Hemes were then extracted twice from these samples with 20 ml of acidified acetone (2.5 ml of 31% HCl/100 ml acetone), the pooled extracts were flash evaporated and the hemes dissolved in 5 ml of alkaline pyridine (pyridine–1 *M* NaOH-water, 5:1:5 by volume, pH 13). An aliquot was taken for the determination of hemes as described by Greim *et al.* (8) and 100 μl was added to 10 ml of toluene-Omnifluor for determination of radioactivity in a Packard Tricarb Model 2425 scintillation spectrometer. Quench data were analyzed by a Control Data Corporation 6400 computer.

The turnover of cytochrome P_{450} apoprotein in selenium deficient animals was assessed in other animals from the two dietary groups. Microsomal fractions were prepared from phenobarbital-injected and saline-injected selenium deficient animals. The microsomal fractions were washed by the

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od of Weihing *et al.* (9) to remove bed and intracisternal proteins and l amounts (50 μg) of sodium dodecyl e (SDS) solubilized microsomal pro- from these animals were applied to a polyacrylamide-SDS-6 *M* urea slab rior to electrophoresis according to the od of Laemmli (10). The gel was fixed stained in Coomassie blue 0.1% in % trichloroacetic acid, 50% methanol ight and destained in 10% acetic acid. entative identification of the protein of wt 50,000-60,000 as cytochrome P_{450} rotein within this system has been de- ed previously (11) and the problems iated with identification discussed

e double isotope-polyacrylamide gel rophoresis technique (13) was utilized eviously described (11) to examine the t of phenobarbital administration on ynthesis of cytochrome P_{450} apoprotein lenium deficient rats. For 5 days prior crifice, one selenium deficient rat was phenobarbital (80 mg/kg/day) while ier received 0.9% NaCl only. On the day of treatment, and after a 12-hr fast, henobarbital treated animal received Ci of [4,5-^{3}H]leucine (Amersham Ra- iemical Centre, 1 Ci/mmol) by intra- oneal injection while the other saline- ed animal simultaneously received 50 of [^{14}C]leucine (Amersham Radi- mical Centre, 348 mCi/mmole). Six s later both animals were killed by guination and a microsomal fraction ned by differential centrifugation of ombined liver homogenate. The micro- s were washed (9), suspended in 1% and 7.5% preparative polyacrylamide electrophoresis performed as above. r overnight freezing the gel was sliced versely into 1.5-mm consecutive slices he ^{3}H and ^{14}C radioactivity in each slice its relationship to Coomassie blue ed bands was determined as previously ibed (11).

ta were analyzed where appropriate by way analysis of variance with replica- (14).

ults. Basal hepatic heme content and esis in the selenium deficient rats did ppreciably differ from the levels in selenium replete animals. However, in the selenium deficient animals hepatic heme synthesis did not increase with phenobarbital treatment to the extent documented in selenium replete rats. Total hepatic heme content (determined as mn*M*/total liver homogenate) increased to 168% of the control value in selenium replete phenobarbital treated rats, while a 10% increase only was observed in the selenium deficient group (Fig. 1). Changes in δ-amino-[^{14}C]levulinic acid incorporation paralleled those of the hepatic heme content. Thus, δ-amino-[^{14}C]levulinic acid incorporation into heme in the selenium replete phenobarbital animals was 158% of control, but no increase was demonstrable in the selenium deficient rats.

Figure 2 illustrates the microsomal heme content and δ-amino-[^{14}C]levulinic acid incorporation following phenobarbital treatment. After correction for inefficiency or incompleteness of microsomal recovery, determined by glucose-6-phosphatase recovery (13), microsomal heme accounted for 60–70% of total hepatic heme. Total microsomal heme content and radioactive incorporation were increased by phenobarbital administration in the selenium replete animals (respectively, 148 and 145% of control, Fig. 2). In contrast, this increase was appreciably less apparent in the selenium deficient animals treated with phenobarbital (values, respectively, 112 and 114% of con-

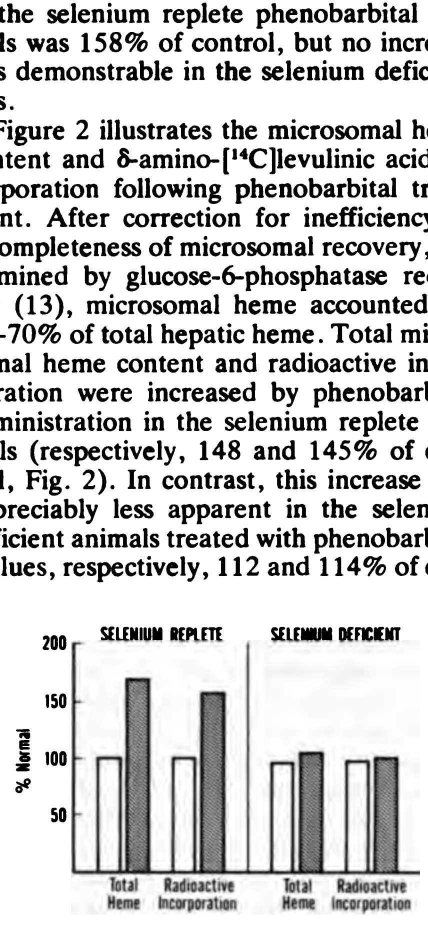

FIG. 1. Total hepatic heme and δ-amino-[^{14}C]levulinic acid incorporation in selenium replete and deficient rats. Heme was extracted and measured as described under methods. Each value, expressed as a percentage of selenium replete control, represents the mean of two animals. Clear bars, saline treated; hatched bars, 5 day phenobarbital treated (80 mg/kg/day).

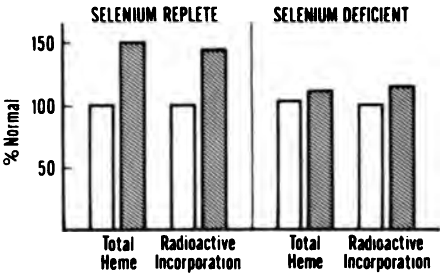

FIG. 2. Microsomal heme content and Δ-amino-[^{14}C]levulinic acid incorporation in selenium replete and deficient rats. Heme was extracted and measured as described under methods. Each value, the mean from two animals, is expressed as a percentage of selenium replete control (960 nm *M* heme/liver; 0.7×10^6 dpm/liver). Clear bars, saline treated; hatched bars, phenobarbital treated.

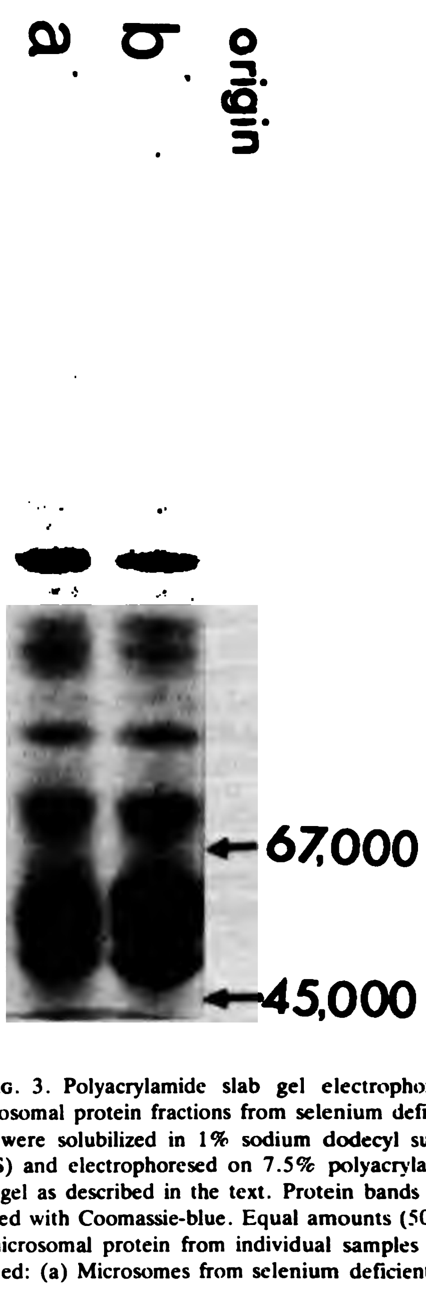

FIG. 3. Polyacrylamide slab gel electrophoresis. Microsomal protein fractions from selenium deficient rats were solubilized in 1% sodium dodecyl sulfate (SDS) and electrophoresed on 7.5% polyacrylamide slab gel as described in the text. Protein bands were stained with Coomassie-blue. Equal amounts (50 μg) of microsomal protein from individual samples were applied: (a) Microsomes from selenium deficient, saline treated animal, (b) Microsomes from selenium deficient animal treated for 5 days with phenobarbital 80 mg mg/kg/day. Molecular weight markers 67,000 (albumin), 45,000 (ovalbumin).

trol, Fig. 2). The value of the interaction term for microsomal heme and radioisotope incorporation between selenium deficient and replete animals treated with phenobarbital was significant ($P < 0.01$) in each case. While hepatic incorporation of δ-amino-[^{14}C]levulinic acid in the selenium deficient rats was not augmented by phenobarbital pretreatment, the specific activity of hepatic heme did not differ between the selenium replete and deficient groups (250–290 dpm/nmole of heme). In all animals the specific activity of heme from microsomal fractions was greater than that of the total hepatic homogenate (800 dpm/mmole of heme).

Figure 3 demonstrates that even in the selenium deficient rat there is an appreciable increase in the density of staining of protein migrating in the mol wt 50,000–60,000 region of a polyacrylamide gel after phenobarbital treatment. Cytochrome P_{450} apoprotein represents a substantial proportion (estimated as 50%, (13)) of polypeptides in this region and an identical increase in staining density after phenobarbital treatment in normal rats suggests that selenium deficiency may not impair the inductive response of cytochrome P_{450} apoprotein.

This impaired response in heme synthesis together with a normal pattern of increase in gel protein staining indicated that discoordinated synthesis of apoprotein and heme was occurring in the phenobarbital treated selenium deficient rats. We therefore investigated the effect of phenobarbital on synthesis rate of apoprotein cytochrome P_{450} in selenium deficiency by a double isotope method. Figure 4 demonstrates a marked increase in ^{3}H/^{14}C ratios in the mol wt

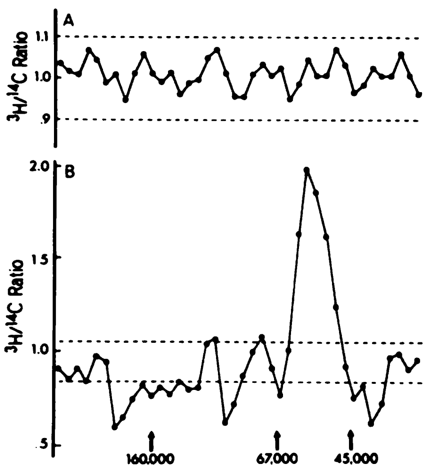

FIG. 4. Effect of phenobarbital on the relative rate of synthesis of apoprotein cytochrome P_{450} in selenium deficient rats. A, (upper panel) control experiment. Both ^{14}C- and ^{3}H-labeled leucine simultaneously delivered to untreated control rats. ^{3}H/^{14}C ratio for each gel slice was divided by homogenate ^{3}H/^{14}C ratio[4]. Dotted horizontal lines represent 95% confidence limits for variation from mean ratio. B, (lower panel) [^{14}C]leucine was administered to saline treated selenium deficient rat, [^{3}]leucine simultaneously administered to phenobarbital treated selenium deficient rat. Animals were killed 6 hr later and microsomes prepared from the combined liver homogenate were electrophoresed on 7.5% polyacrylamide (see text). Molecular weight markers 45,000 (ovalbumin), 67,000 (albumin), 160,000 (gammaglobulin). Dotted horizontal lines represent 95% confidence limits established in control experiment.

50,000-60,000 region of the gel similar to that previously demonstrated in normal phenobarbital treated rats (11).

Discussion. The determination of cytochrome P_{450} involves the spectrophotometric measurement of the heme moiety, and normally the apoprotein content is not determined. Siekevitz (15) recently presented evidence that in fetal and neonatal rats there is an apparent discoordination in the appearance of the heme and apoprotein moieties of cytochrome P_{450}. Thus, the previous demonstration (4) of impaired inducibility of cytochrome P_{450} by phenobarbital in selenium deficient rats, with, however, normal inducibility of the flavoprotein NADPH cytochrome c reductase, suggested that a comparable discoordination of synthesis of these two moieties may be present. An alternative explanation that heme transport to the microsomes rather than heme synthesis was impaired seemed unlikely in view of the observation of Murty *et al*. (16) that vitamin E deficiency impaired both bone marrow and hepatic heme synthesis.

The present study confirms the concept of discoordinated synthesis of heme and apoprotein in selenium deficient phenobarbital treated rats. Thus, the anticipated increase in δ-amino-[^{14}C]levulinic acid incorporation was not apparent in the selenium deficient rats, and the observation that total hepatic radioisotope incorporation as well as microsomal isotope incorporation were impaired indicated that the defect is one of impaired heme synthesis rather than a defect in intracellular transport of heme.

Two approaches were utilized to demonstrate that in the selenium deficient rats exposure to phenobarbital was associated with an increase in cytochrome P_{450} apoprotein. Thus, both polyacrylamide gel staining and radio-active leucine incorporation indicated that cytochrome P_{450} apoprotein induction was not impaired by selenium deficiency. In view of the present impossibility of the certain identification of cytochrome P_{450} apoprotein(s) this conclusion must be qualified. It may be stated, however, that the induction of proteins or polypeptides of mol wt 50,000-60,000 did not appear quantitatively different in selenium deficient rats and selenium replete controls, and since cytochrome P_{450} apoprotein represents the major protein in this molecular weight range in our polyacrylamide gel system (13) a workable extrapolation seems justifiable.

Thus, in the selenium deficient animal the synthesis of the heme and apoprotein moieties of cytochrome P_{450} do not respond identically to phenobarbital induction. The recent demonstration (15) of discoordinated synthesis of heme and apoprotein in the neonate argues against the proposal that induction of heme biosynthesis leads in turn to enhanced cytochrome P_{450} synthesis (17, 18), but rather favors a primary effect of phenobarbital in increasing P_{450} synthesis with limited availability of heme resulting in excess apocytochrome P_{450} (19, 20).

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Effect of Leukocytic Endogenous Mediator (LEM) on Zinc Absorption in the Rat (39443)

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Hypozincemia, hypozincuria, and the increased hepatic uptake of zinc are characteristic responses of the mammalian host during many infections, endotoxemia, and other inflammatory stresses (1-4). Recently, it was postulated that leukocytic endogenous mediator (LEM), a heat labile, low molecular weight protein released by polymorphonuclear (PMN) leukocytes, may be a key intermediate in stimulating the above alterations in zinc metabolism during inflammation (5-7).

More recently, Pekarek and Evans (8) demonstrated that acute infection and endotoxemia in rats enhanced the intestinal absorption of zinc with a significant accumulation of zinc in the livers of the stressed animals. While the mechanism for altered zinc absorption during inflammation remains uncertain, the present investigation was undertaken to determine whether LEM could be responsible, in part, for the increased intestinal absorption and hepatic uptake and retention of zinc observed during acute inflammatory stresses.

Materials and methods. Healthy adult male New Zealand white rabbits, weighing 2-3 kg, and male Sprague-Dawley rats, weighing 150-175 g, were used in our study. The LEM was prepared by infusing rabbits intraperitoneally (ip) with approximately 300 ml of a sterile pyrogen-free normal saline solution containing 0.2% shellfish glycogen (Mann Research Laboratories).[1] Peritoneal exudates were collected aseptically 14 hr after infusion, and the PMN leukocytes were obtained by centrifugation. The harvested cells were washed twice with cold saline; resuspended at 1 × 10^8 cell/ml in sterile pyrogen-free normal saline which contained 10 U/ml of heparin, 150,000 U/liter of penicillin G, and 0.4 g/liter of streptomycin; and incubated at 37° for 2 hr with gentle shaking (5, 6). Following incubation, the cells were removed by centrifugation; the supernatant represented the crude LEM preparation.

The effect of LEM on zinc metabolism was tested by injecting each of a group of rats ip with 1 ml of LEM preparation. Each of another group of rats was injected ip with an equal aliquot of the LEM preparation that was heat inactivated (90° for 30 min). This was done as a control to insure that the observed changes in zinc metabolism were not due to any contaminating heat-stable endotoxins. Each rat of a third group received an equal volume of sterile pyrogen-free normal saline ip and the group served as an additional set of controls.

Six hours after the administration of the above materials, zinc absorption was studied by diluting carrier-free ^{65}Zn (International Chemical and Nuclear Corp.)[1] with cold $ZnCl_2$ in normal saline to give a final concentration of 1.3 μg Zn^{2+}/ml. All rats in each test group received a 0.5-ml dose of the radiolabeled Zn by gastric intubation. One hour following the administration of the isotope, the rats were decapitated, blood was collected in heparinized tubes, and the entire gastrointestinal tracts were removed. The radioactivity in the carcasses, the stomachs and remaining intestinal tracts were measured in a whole-body ARMAC Scintillation Detector (Model 446, Packard Instrument Co., Inc.)[1] by an Auto Gamma Spectrometer (Model 2001, Packard Instrument Co., Inc.)[1]. Zinc absorption was measured 1 hr after the oral administration of the isotope in order to minimize the secretion of zinc back into the intestine (8). The

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[1] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

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amount of isotope absorbed into the body was calculated as follows:

$$\% \text{ absorbed} = \frac{\text{carcass } ^{65}\text{Zn}}{\text{carcass} + \text{intestine-stomach}} .$$

In addition, the livers were removed and the amount of isotope taken up and retained by the liver was expressed in a similar manner:

$$\% \text{ uptake} = \frac{\text{liver } ^{65}\text{Zn}}{\text{carcass} + \text{liver}} .$$

Plasma zinc concentrations were determined by atomic absorption spectrophotometry.

Results. Based on the amount of isotope that reached the intestine from the stomach, ^{65}Zn absorption was significantly ($P < .005$) higher in the LEM-treated rats than in either the saline or heat inactivated LEM-treated controls (Table I). In addition, LEM produced a significant increase in the hepatic uptake of ^{65}Zn and decrease in plasma zinc concentrations. While the LEM preparation was highly active, the heat treated (90° for 30 min) LEM failed to produce any changes in zinc metabolism in the rat; thus, showing that the LEM preparation used in this study was apparently free of any contaminating heat-stable endotoxins.

Discussion. Evans *et al.* (9, 10) have shown that zinc absorption is inversely proportional to the intestinal and plasma zinc concentrations. More specifically, Richards and Cousins (11) have demonstrated that zinc absorption is in part regulated by the concentration of metallothionein, a zinc binding protein, in the intestinal mucosa. metallothionein synthesis, in turn, is regulated by the amount of zinc present in the intestinal cells. Thus, when the plasma zinc content is low, less zinc enters the intestinal cells from the lamina propria resulting in a concomitant reduction in metallothionein synthesis. In turn, more zinc is transported across the cells of the intestinal mucosa via a low molecular weight ligand to binding sites on the basolateral plasma membrane where it is removed by metal binding plasma proteins (12).

In this regard, acute infection and endotoxemia have been shown to produce significant alterations in normal zinc metabolism, resulting in a rapid flux of zinc into the liver, a lowering of the plasma zinc concentration and an increase in the intestinal absorption of this metal (8). Thus, the lowering of plasma zinc due to the rapid redistribution of this metal to the liver and possibly to other tissues would explain the increase in zinc absorption observed during the inflammatory process according to our present knowledge of zinc homeostasis.

The data from the present study demonstrate that a factor released from PMN leukocytes also initiates these changes in zinc metabolism when PMN leukocytes are administered to a normal rat. This factor has been shown to be present in the plasma of patients with a variety of acute infections (13, 14) and in experimentally infected laboratory animals (5). While LEM may be a key intermediate in altering zinc homeostasis during the inflammatory process, its mechanism of action remains somewhat obscure. However, LEM also has been shown to induce the hepatic uptake of plasma free amino acids (15) and the increase synthesis and/or release of various acute phase glycoproteins (16, 17). Since zinc has been shown to be essential for RNA, DNA and certain aspects of protein synthesis (18–20), there may be a greater requirement for this metal in the liver during inflammation. While the functional role of zinc in the liver and the mechanism by which LEM acts to increase the hepatic zinc content during inflammation are still unknown, the fact remains that

TABLE 1. EFFECT OF LEM ON ZINC ABSORPTION AND DISTRIBUTION IN THE RAT

Treatment of rat (1 ml ip)	Carcass Zn (% absorbed)[a]	Liver Zn (% absorbed)[a]	Plasma Zn (μg/100 ml)[a]
LEM	38.1 ± 1.9[b]	52.1 ± 2.4[b]	36 ± 2[b]
Heat inactivated LEM	22.2 ± 3.1	38.8 ± 2.8	124 ± 5
Saline control	22.9 ± 1.8	42.3 ± 0.9	125 ± 5

[a] Minimum of six animals ± SE.

[b] Significantly different from control groups ($P < .005$).

ıe inflammatory process causes a redistriution of zinc within the tissue of the body ith a concomitant increase in the intestinal bsorption of this metal.

Summary. Leukocytic endogenous meiator (LEM) was shown to produce a significant increase in the intestinal absorption f zinc within 7 hr after its administration to ormal rats. Consistent with earlier studies, EM also produced a significant decrease in ıe plasma zinc concentration and an inreased hepatic uptake of this metal which ırther demonstrates that LEM may be a ey intermediate in altering zinc homeostas during inflammation.

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The Hydrolysis of Biologically Active Peptides by Bovine Lung Tissue Factor (Thromboplastin)[1] (39444)

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Tissue extracts from almost all mammalian organs contain tissue factor activity which can promote blood coagulation via the extrinsic system (1). Nemerson and Pitlick (2) solubilized tissue factor from bovine lung and brain and purified the protein component. Delipidation and solubilization resulted in a loss of coagulant activity which could be restored by recombining the apoprotein with appropriate phospholipids (2, 3). Pitlick *et al.* (4) observed that particulate as well as delipidated tissue factor possessed peptidase activity. Tissue factor could hydrolyze synthetic di- and tripeptides and aminoacyl-β-naphthylamides and release a number of free amino acids from the oxidized B chain of insulin.

It was of interest to determine if tissue factor peptidase activity might serve the function of metabolizing physiologically important peptides. This paper presents data demonstrating the ability of relipidated and apoprotein tissue factor from bovine lung to hydrolyze a number of vasoactive peptides and hypothalamic and neurohypophyseal hormones. Preliminary reports have appeared elsewhere (5, 6).

Materials. Dansyl chloride and dansyl amino acids were purchased from Sigma Chemical Co. Additional dansyl amino acid and peptide markers were prepared from the respective amino acids and peptides by the method of Gray (7). Polyamide layer sheets, prepared by the Cheng Chin Trading Co., were purchased through Gallard-Schlesinger. Synthetic peptides were obtained as follows: substance P (Beckman Instruments, Spinco Division); oxytocin (A grade, Calbiochem); bradykinin, Lys-bradykinin, Met-Lys-bradykinin, [Asp^1,Ile^5]-angiotensin I, [Asp^1,Ile^5]-angiotensin II, bradykinin potentiating pentapeptide (BPP$_{5a}$)[3] and nonapeptide (BPP$_{9a}$) from *Bothrops jararaca*, LH-RH, TRF, and human fibrinopeptide A (Spectrum Medical Industries); MIF and additional quantities of LH-RH and oxytocin (Dr. Maurice Manning, Medical College of Ohio at Toledo); [Sar^1,Ile^5]-angiotensin II and [Me$_2$Gly^1,Ile^5]-angiotensin II (Dr. M. C. Khosla, Cleveland Clinic Foundation). All peptides were assayed for purity, for amino-terminal residues, and for amino acid composition by the dansylation method (7). Additional materials were obtained as follows: ninhydrin (Schwarz/Mann); constant-boiling hydrochloric acid (Pierce Chemical); 2-mercaptoethanol (Eastman). All other chemicals were reagent grade.

Mixed brain phospholipids were prepared essentially in the manner described by Nemerson (8). Micellization (3) was performed in 0.5% sodium deoxycholate.

Tissue factor purification. Tissue factor apoprotein was purified from bovine lung by the method of Nemerson and Pitlick (2), using hydrolysis of alanyl-β-naphthylamide as the enzyme assay (4). Disc gel electro-

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[1] This work is taken from dissertations to be submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy by W.H.S. from Ohio State University and D.E.B. from Bowling Green State University.

[2] Research Associateship Awardee of the Faculty Research Committee of Bowling Green State University.

[3] Abbreviations: LH-RH, luteinizing hormone-releasing hormone; MIF, melanocyte stimulating hormone-release-inhibiting factor (Pro-Leu-Gly-NH$_2$); TRF, thyrotropin-releasing factor; [Sar^1]-angiotensin II, [1-*N*-methylglycine]-angiotensin II; [Me$_2$Gly^1]-angiotensin II, [1-*N*,*N*-dimethylglycine]-angiotensin II; BPP$_{5a}$, bradykinin potentiating peptide (pentapeptide from *Bothrops jararaca*); BPP$_{9a}$, bradykinin potentiating peptide (nonapeptide from *B. jararaca*); DNS-, dansyl (5-dimethylaminonaphthalene-1-sulfonyl).

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is of purified tissue factor apoprotein dard gels (9) revealed a single major band which barely entered the sepa- gel and a minor contaminant running e front.

idation of tissue factor apoprotein. d tissue factor apoprotein was relipi- with mixed brain phospholipids ac- to Nemerson (10) at a ratio of 5.0 ipid/mg of protein. Relipidated tissue contained coagulant activity as mea- with the one-stage prothrombin time 11, 12) while apoprotein was com- devoid of coagulant activity.

idase activity. Relipidated tissue fac- d tissue factor apoprotein control ssayed for their ability to hydrolyze a of physiologically active peptides by ing the increase in the appearance of nino groups with time by the ninhy- ethod (13). Assay mixtures consisted ml of enzyme solution (15 μg pro- d 0.1 ml of 0.5 m*M* substrate in 0.02 ssium phosphate, pH 6.8. The mix- ere incubated at 37° and 25-μl ali- vere removed at 1 min and 2.5, 5.0, 5 hr and inactivated with 1 ml of rin.

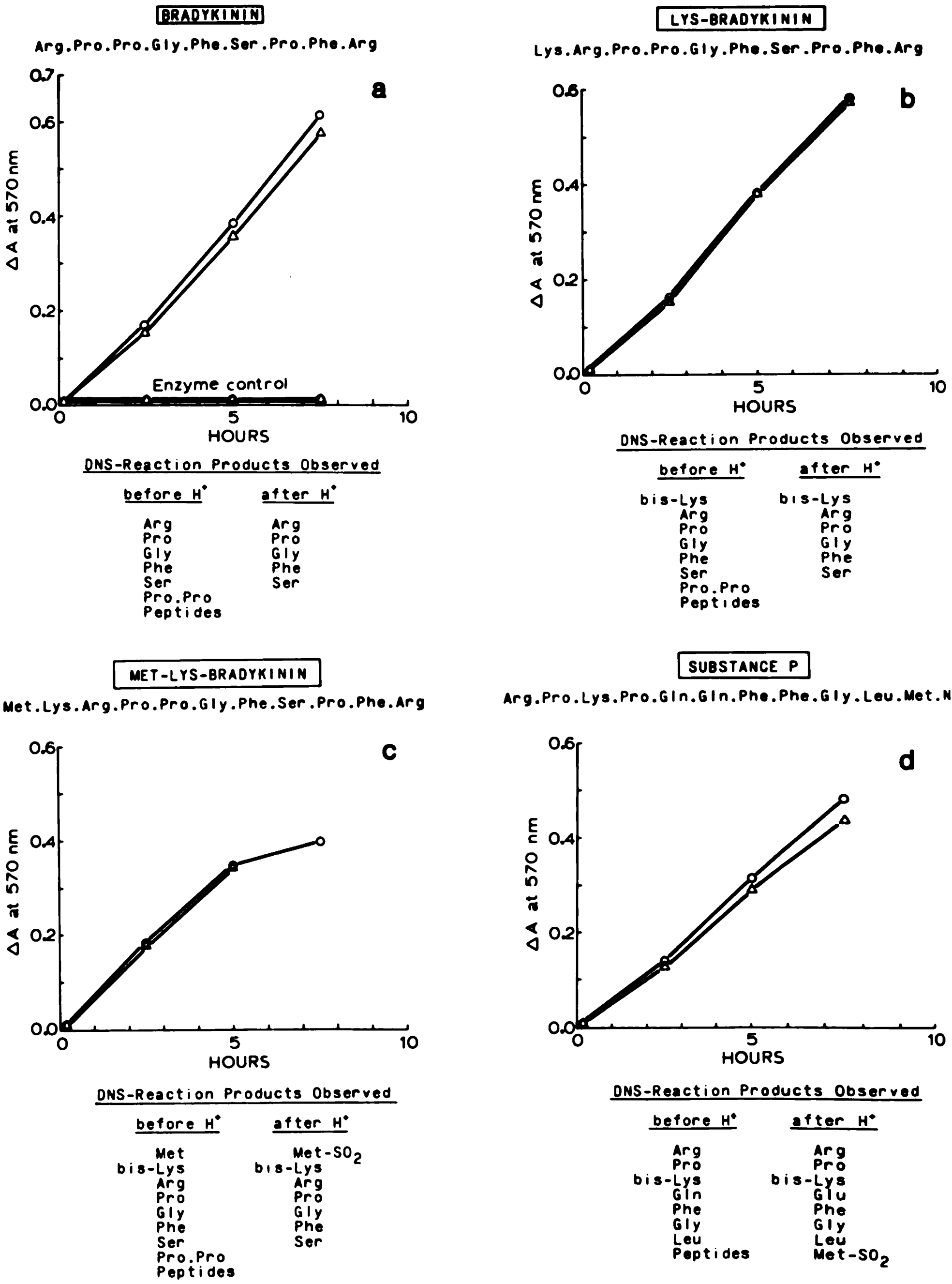
sylation and chromatography of tissue peptide incubation mixtures. Tissue peptide incubation mixtures were ted by the method of Gray (7) and tographed in order to identify reac- oducts. Relipidated tissue factor and actor apoprotein (15 μg in 0.05 ml) ncubated at 37° with 0.1 ml of 0.5 bstrate in 0.02 *M* potassium phos- pH 6.8. Aliquots (10 μl) were re- from the incubation mixtures at 1 d 2.5, 5.0, and 7.5 hr and placed in f 0.2 *M* sodium bicarbonate to which mediately added 25 μl of dansyl chlo- agent (5 mg/ml in acetone). After tion of the dansylation reaction (1 hr , samples were dried *in vacuo* and suspended in 25 μl of acetone–0.1 *N* cid (3:2). A portion of each dansyl- action mixture (1.5 μl) was chromat- ed in two dimensions on 7.5 × 7.5- yamide layer sheets using Solvent I -90% formic acid, 200:3) and Sol- (benzene–glacial acetic acid, 9:1). cond portion of each dansylated mix- as acid hydrolyzed by heating the sample with constant-boiling HCl in a sealed tube at 105° for 9–16 hr. After removal of the HCl *in vacuo* over NaOH pellets, samples were resuspended and chromatographed on polyamide layers as described above.

Fluorescent spots were detected on chromatograms with an ultraviolet lamp at 254 nm and identified by comparing R_f values with R_f values of standard dansyl amino acids and peptides chromatographed separately in each solvent. In most cases, the identities of fluorescent spots were confirmed by chromatographing samples containing selected internal dansyl amino acid standards. The individual synthetic peptide substrates and the enzyme solutions were dansylated separately and chromatographed, both with and without prior acid hydrolysis. These chromatograms served as controls. Qualitative amino acid analysis of each peptide was determined by chromatographing a sample of the peptide which had first been acid hydrolyzed and then dansylated.

Chemical methods. Protein concentration was determined by the method of Lowry *et al.* (14). The phosphorous content of mixed brain phospholipids was determined by the method of Chen *et al.* (15).

Results. Effect of tissue factor on physiologically active peptides. Figure 1 shows that both relipidated and apoprotein tissue factor extensively hydrolyzed bradykinin, Lys-bradykinin, Met-Lys-bradykinin, substance P, angiotensin I, angiotensin II, and fibrinopeptide A, while acting more slowly on [Sar^1]-angiotensin II, [Me$_2$Gly^1]-angiotensin II, and bradykinin potentiating pentapeptide from *B. jararaca* (BPP$_{5a}$). LH-RH, MIF, and oxytocin were also hydrolyzed quite slowly while TRF and bradykinin potentiating nonapeptide from *B. jararaca* (BPP$_{9a}$) were not hydrolyzed at all. There was no difference in the rate of hydrolysis of any of the peptides by either relipidated tissue factor or tissue factor apoprotein under the conditions of our assay. Dansylated enzyme-peptide reaction mixtures chromatographed after various times of incubation indicated that relipidated tissue factor and tissue factor apoprotein degraded peptides by identical mechanisms.

Bradykinin. Figure 1a shows that 50



BRADYKININ

Arg.Pro.Pro.Gly.Phe.Ser.Pro.Phe.Arg

DNS-Reaction Products Observed

before H$^+$	after H$^+$
Arg	Arg
Pro	Pro
Gly	Gly
Phe	Phe
Ser	Ser
Pro.Pro	
Peptides	

LYS-BRADYKININ

Lys.Arg.Pro.Pro.Gly.Phe.Ser.Pro.Phe.Arg

DNS-Reaction Products Observed

before H$^+$	after H$^+$
bis-Lys	bis-Lys
Arg	Arg
Pro	Pro
Gly	Gly
Phe	Phe
Ser	Ser
Pro.Pro	
Peptides	

MET-LYS-BRADYKININ

Met.Lys.Arg.Pro.Pro.Gly.Phe.Ser.Pro.Phe.Arg

DNS-Reaction Products Observed

before H$^+$	after H$^+$
Met	Met-SO$_2$
bis-Lys	bis-Lys
Arg	Arg
Pro	Pro
Gly	Gly
Phe	Phe
Ser	Ser
Pro.Pro	
Peptides	

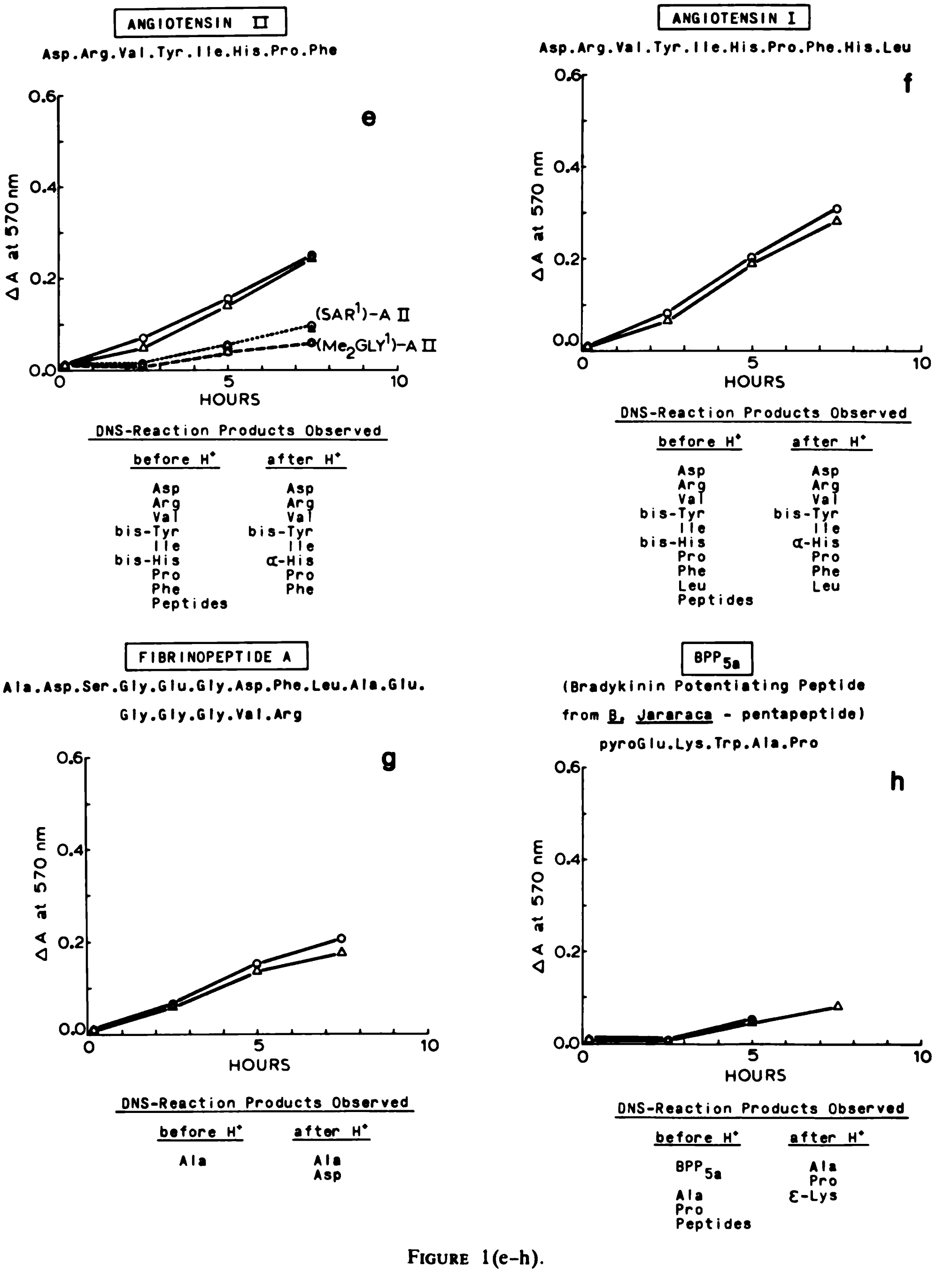
SUBSTANCE P

Arg.Pro.Lys.Pro.Gln.Gln.Phe.Phe.Gly.Leu.Met.NH$_2$

DNS-Reaction Products Observed

before H$^+$	after H$^+$
Arg	Arg
Pro	Pro
bis-Lys	bis-Lys
Gln	Glu
Phe	Phe
Gly	Gly
Leu	Leu
Peptides	Met-SO$_2$

FIG. 1. The effect of relipidated and apoprotein bovine lung tissue factor on physiologically active peptides. Relipidated tissue factor or tissue factor apoprotein (15 μg in 0.05 ml) was incubated at 37° with 0.1 ml of 0.5 m*M* substrate in 0.02 *M* potassium phosphate, pH 6.8. Aliquots (25 μl) were removed at 1 min and 2.5, 5.0, and 7.5 hr and inactivated with 1 ml of ninhydrin. The ninhydrin tubes were developed by heating in a boiling-water bath for 20 min. After dilution with 1 ml of 1-propanol-H$_2$O (1:1), samples were read at 570 nm in a Turner Model 330 spectrophotometer with an Instafill attachment. The absorbance at 1 min was subtracted from the values at other time periods. Graphs in the figure indicate Δ*A* as a function of incubation time (hours). A Δ*A* = 0.097 represents 50 nmoles of free amino groups liberated from the 50 nmoles of substrate (based on the color yield of leucine), indicating approximately one bond cleaved. Δ, Relipidated; O, apoprotein. Reaction products were identified by dansylating aliquots of incubation mixtures by the method of Gray (7) at the different time periods given above. The dansylated mixtures were chromatographed on polyamide layer sheets either before acid hydrolysis (before H$^+$) or else after acid hydrolysis to release terminal DNS-amino acids from peptides (after H$^+$). The figure indicates those dansyl reaction products observed after 7.5 hr. The products were identical for both relipidated and apoprotein tissue factor. Bis- indicates that the side chain functional group as well as the α-amino group of the amino acid was dansylated. α-His and ε-Lys indicate that only the α-amino group of histidine and the ε-amino oup of lysine, respectively, were dansylated.



ANGIOTENSIN II

Asp.Arg.Val.Tyr.Ile.His.Pro.Phe

DNS-Reaction Products Observed

before H⁺	after H⁺
Asp	Asp
Arg	Arg
Val	Val
bis-Tyr	bis-Tyr
Ile	Ile
bis-His	α-His
Pro	Pro
Phe	Phe
Peptides	

ANGIOTENSIN I

Asp.Arg.Val.Tyr.Ile.His.Pro.Phe.His.Leu

DNS-Reaction Products Observed

before H⁺	after H⁺
Asp	Asp
Arg	Arg
Val	Val
bis-Tyr	bis-Tyr
Ile	Ile
bis-His	α-His
Pro	Pro
Phe	Phe
Leu	Leu
Peptides	

FIBRINOPEPTIDE A

Ala.Asp.Ser.Gly.Glu.Gly.Asp.Phe.Leu.Ala.Glu.
Gly.Gly.Gly.Val.Arg

DNS-Reaction Products Observed

before H⁺	after H⁺
Ala	Ala
	Asp

BPP_{5a}

(Bradykinin Potentiating Peptide from B. Jararaca - pentapeptide)

pyroGlu.Lys.Trp.Ala.Pro

DNS-Reaction Products Observed

before H⁺	after H⁺
BPP_{5a}	Ala
Ala	Pro
Pro	ε-Lys
Peptides	

FIGURE 1(e-h).

nmoles of bradykinin was extensively hydrolyzed by 15 μg of either relipidated or apoprotein tissue factor as measured by the ninhydrin method. Both enzyme preparations, when incubated alone, showed no increase in ninhydrin color. The dansylated enzyme-bradykinin incubation mixtures, which were acid hydrolyzed to release terminal DNS-amino acids from intermediate peptides and then chromatographed, also indicated extensive degradation of the peptide. Since each dansyl amino acid observed indicated hydrolysis of the peptide bond on the amino side of that residue, it was concluded that tissue factor hydrolyzed bradykinin at the Pro^3Gly^4 and Phe^5Ser^6 bonds and at the amino side of at least one of the three prolines and at least one of the two phenylalanines. The intensity of the DNS-Arg spot increased with increasing incubation time suggesting that, in addition to the constant level of DNS-Arg contributed by the N-terminal residue, DNS-Arg was also being contributed by arginine released from the C terminus. Thus, tissue factor also hydrolyzed the Phe^8Arg^9 bond. Chromatograms of nonacid-hydrolyzed, dansylated incubation mixtures, which indicated the actual composition of the reaction mixtures, revealed several spots having R_f values identical to several free dansyl amino acids. The presence of free DNS-Gly and DNS-Ser in the mixture suggested that the Gly^4Phe^5 and Ser^6Phe^7 bonds were also hydrolyzed. Intermediate DNS-peptides were also observed. One peptide had an N-terminal DNS-Pro and cochromatographed with authentic DNS-Pro-Pro. This indicated that tissue factor hydrolyzed the Arg^1Pro^2 bond. A second peptide had an N-terminal DNS-Ser. The intensities of free DNS-Ser and DNS-Pro spots did not increase as rapidly with time as other free DNS-amino acids confirming that tissue factor hydrolyzed the Ser^6Pro^7 and Pro^2Pro^3 bonds more slowly than other bonds in the molecule. In summary, no fewer than six bonds were cleaved in bradykinin.

Lys-bradykinin. Figure 1b shows that relipidated and apoprotein tissue factor hydrolyzed Lys-bradykinin at about the same rate as bradykinin. Dansyl chromatograms indicated that the mechanism of degradation of Lys-bradykinin was also similar to that of bradykinin. The chromatograms of the nonacid-hydrolyzed dansylated reaction mixtures revealed a spot with R_f values identical to free bis-DNS-Lys, suggesting that tissue factor also hydrolyzed the Lys^1Arg^2 bond.

Met-Lys-bradykinin. Met-Lys-bradykinin was hydrolyzed by tissue factor at a rate and by a mechanism similar to bradykinin and Lys-bradykinin (Fig. 1c). In this case, the Met^1Lys^2 bond was also hydrolyzed.

Substance P. Figure 1d indicates that tissue factor extensively hydrolyzed substance P, potentially cleaving every peptide bond in the molecule. Chromatograms of dansylated reaction mixtures at different periods of incubation suggested that the Pro^2Lys^3, Gln^5Gln^6, Gln^6Phe^7, and Phe^7Phe^8 bonds were hydrolyzed rapidly while Arg^1Pro^2, Lys^3Pro^4, Phe^8Gly^9, and $Leu^{10}Met^{11}$ bonds were hydrolyzed more slowly. The Gly^9Leu^{10} bond was cleaved at an intermediate rate and the Met-NH_2 amide bond was not hydrolyzed at all. It was impossible from the present data to determine whether or not the Pro^4Gln^5 bond was cleaved.

Angiotensin II. [Asp^1Ile^5]-angiotensin II was hydrolyzed by tissue factor at a rate less than one-half that of bradykinin (Fig. 1e). However, dansyl chromatograms indicated that tissue factor could degrade angiotensin II to constituent amino acids. The chromatograms of the nonacid-hydrolyzed dansylated mixtures revealed the presence of a major intermediate peptide which had a terminal DNS-His. The slow appearance of free DNS-His and DNS-Pro confirmed that the His^6Pro^7 bond was more resistant to cleavage than other bonds in the molecule.

[Sar^1]-angiotensin II and [Me_2Gly^1]-angiotensin II. Figure 1e also shows that [Sar^1]-angiotensin II and [Me_2Gly^1]-angiotensin II were hydrolyzed at rates 40 and 25%, respectively, as fast as [Asp^1]-angiotensin II. Dansyl chromatographic data indicated that major cleavage in these analogs occurred at Pro^7Phe^8 with the release of free phenylalanine. Although the remainder of the molecule could be degraded to constituent amino acids, the rate was greatly retarded compared to [Asp^1]-angiotensin II.

Angiotensin I. Angiotensin I was hydrolyzed by tissue factor at a rate slightly greater than angiotensin II and was also cleaved at every peptide bond (Fig. 1f). The chromatograms of the non-acid-hydrolyzed dansylated reaction mixtures revealed two major intermediate peptides, both of which had N-terminal DNS-His residues. One of the peptides had the same R_f value as a similar spot in angiotensin II chromatograms and was presumably His-Pro. The other peptide was seen only in angiotensin I chromatograms and was probably His-Leu. The slow appearance of free DNS-His, DNS-Pro, and DNS-Leu confirmed these ments and indicated that the His^{6}Pro^7 is^{9}Leu^{10} bonds were more resistant to ge than other bonds in the molecule. was no evidence to suggest, however, ssue factor initially released His-Leu m angiotensin II before further de- g the active portion of the molecule.

nan fibrinopeptide A. Figure 1g shows ssue factor hydrolyzed fibrinopeptide at a rate which suggested only limited ge of this 16-amino-acid peptide. natograms of acid-hydrolyzed dansyl- eaction mixtures revealed the pres- of DNS-Asp, suggesting cleavage of the Ala^{1}Asp^2 or Gly^{6}Asp^7 bond or The observed DNS-Ala spot came the N terminus of fibrinopeptide A. pot did not increase in intensity with sing incubation time indicating that u^{9}Ala^{10} bond was not hydrolyzed. omatograms of nonacid-hydrolyzed lated samples revealed only DNS-Ala, ting that alanine was released from terminus. Thus, major cleavage of peptide A by tissue factor occurred at la^{1}Asp^2 bond, although the possibility litional cleavage at Gly^{6}Asp^7 could not minated.

dykinin potentiating pentapeptide from araca (*BPP$_{5a}$*). Figure 1h shows that factor hydrolyzed BPP$_{5a}$ only slowly. l chromatograms suggested that pro- nd alanine were released from the C ius. There was considerably more Ala than DNS-Pro in the acid-hydro- dansylated samples, suggesting that cleavage may have taken place at la^4 followed by slower hydrolysis of sulting dipeptide, Ala-Pro. The nona- drolyzed samples revealed the slow qual appearance of free DNS-Ala and Pro confirming this conclusion. There me evidence that tryptophan could be ed from BPP$_{5a}$ upon extended incuba- ith tissue factor.

dykinin potentiating nonapeptide from raraca (*BPP$_{9a}$*) (*pyroGlu-Trp-Pro- ro-Gln-Ile-Pro-Pro*). Tissue factor did ydrolyze BPP$_{9a}$. Dansyl chromato- revealed no detectable hydrolysis cts.

-RH (*pyroGlu-His-Trp-Ser-Tyr-Gly- rg-Pro-Gly-NH$_2$*). Tissue factor hy- zed LH-RH slowly. Dansyl chromatograms revealed several faint spots which have not yet been firmly identified.

MIF (*Pro-Leu-Gly-NH$_2$*). MIF was also hydrolyzed quite slowly by tissue factor. However, dansyl chromatograms indicated that tissue factor did hydrolyze both peptide bonds and the terminal amide bond. Chromatograms of nonacid-hydrolyzed dansylated reaction mixtures indicated that DNS-MIF was by far the most intense spot, confirming the ninhydrin data. DNS-Pro, DNS-Leu, and DNS-Gly-NH$_2$ were all faint but of equal intensity. This suggested that hydrolysis of one of the peptide bonds occurred with difficulty followed by immediate hydrolysis of the second bond. Hydrolysis of the C-terminal amide bond occurred only after extended periods of incubation.

Oxytocin (*Cy$_s$-Tyr-Ile-Gln-Asn-Cy$_s$-Pro-Leu-Gly-NH$_2$*). Oxytocin was hydrolyzed very slowly by tissue factor. Dansyl chromatograms revealed a variety of faint spots indicating that hydrolysis of oxytocin was possible, albeit with difficulty.

TRF (*pyroGlu-His-Pro-NH$_2$*). Tissue factor did not hydrolyze TRF. Dansyl chromatograms revealed no hydrolysis products.

Effect of 2-mercaptoethanol on relipidated tissue factor hydrolysis of bradykinin. Figure 2 shows that relipidated tissue factor hydrolysis of bradykinin was reduced about 90% in the presence of 20 m*M* 2-mercaptoethanol as measured by the ninhydrin method. Tissue factor apoprotein hydrolysis of bra-

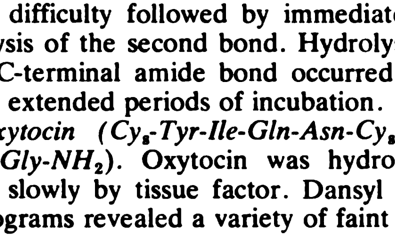


FIG. 2. Effect of 2-mercaptoethanol on the hydrolysis of bradykinin by relipidated tissue factor. Assay mixtures consisted of 0.05 ml relipidated tissue factor (12 μg protein), 0.05 ml of 0.05 m*M* bradykinin in 0.02 *M* potassium phosphate, pH 6.8, and 0.05 ml of 60 m*M* 2-mercaptoethanol in the same buffer (final concentration of 2-mercaptoethanol was 20 m*M*). A control was run in which the 2-mercaptoethanol was replaced by buffer. Mixtures were incubated at 37° and 25 μl aliquots were removed at 1 min., 2.5 hr, and 5 hr and assayed by the ninhydrin method as described in Fig. 1.

dykinin and angiotensin I was also reduced by about 80 and 90%, respectively, in the presence of 20 m*M* 2-mercaptoethanol.

Discussion. The data presented here extend the observations of Pitlick *et al.* (4) by demonstrating that the peptidase activity associated with bovine lung tissue factor can degrade a series of physiologically significant peptides. Since tissue factor represents approximately 1% of the particulate and nonextractable protein of bovine lung (2), the broad specificity of this enzyme toward peptide substrates may implicate it along with other peptidases (16) in a variety of physiological activities other than those related to direct initiation of the extrinsic clotting mechanism.

Tissue factor can readily hydrolyze the kinins and angiotensins *in vitro.* Ryan and co-workers (17-21) reported earlier that bradykinin and angiotensin I are hydrolyzed in the isolated perfused rat lung by enzymes which are probably located near the luminal surface of vascular endothelial cells. Furthermore, the pattern of hydrolysis of peptide bonds of bradykinin and its higher homologs by perfused lung enzymes is essentially identical to that reported for tissue factor herein. Zeldis *et al.* (22), using peroxidase-conjugated antibodies raised against purified tissue factor apoprotein, found that the antigen was located in highest concentration on the plasma membranes of vascular endothelial cells from all tissues studied, including lung. Inasmuch as tissue factor can hydrolyze the kinins and angiotensins *in vitro* and is known to be located at the site of hydrolysis *in vivo,* it is suggested that tissue factor may be one of the enzymes involved in the metabolism of circulating vasoactive peptides.

Although angiotensin-converting enzyme (kininase II) has been implicated in pulmonary metabolism of circulating kinins (23), the known specificity of this enzyme cannot account for all of the cleavages seen in the bradykinin molecule upon passage through the isolated lung. Furthermore, Stewart *et al.* (24) showed that BPP_{5a}, an inhibitor of angiotensin-converting enzyme, could only reduce the pulmonary inactivation of bradykinin in the rat from 98% to a plateau level of 30%. The ability of lung to inactivate 30% of intravenously injected bradykinin in the presence of large doses of BPP_{5a} suggests the presence of another inactivating enzyme, possibly tissue factor. Tissue factor was observed in this study to be greatly inhibited by 2-mercaptoethanol. Similarly, 2-mercaptoethanol destroyed all remaining bradykininase activity in lung and actually potentiated the effect of BPP_{5a}, perhaps by inhibiting its destruction by tissue factor. It is noteworthy that although BPP_{5a} is a more potent inhibitor of angiotensin-converting enzyme than BPP_{9a} *in vitro* (25), it is not as potent *in vivo,* having a much shorter half-life (26). Correspondingly, tissue factor can slowly destroy BPP_{5a}, but leaves BPP_{9a} intact even after a 7.5-hr incubation.

Tissue factor was observed to degrade angiotensin I and II to constituent amino acids. Ryan *et al.* (19, 20) observed that perfused rat lung also hydrolyzed angiotensin I at the His^6Pro^7, Pro^7Phe^8, and Phe^8His^9 bonds and possibly at the Tyr^4Ile^5 and Ile^5His^6 bonds. Additional cleavages probably occurred at the amino terminal end (19) but were not identified. Recently Ryan indicated (27) that rat lung also destroyed 10% of perfused angiotensin II, even though the perfusate showed no reduction in blood pressure response. Although angiotensin-converting enzyme was, undoubtedly, at least partially responsible for cleavage of the Phe^8His^9 bond of angiotensin I, it could not have hydrolyzed any of the remaining bonds in angiotensin I or II (28). It is therefore suggested that tissue factor may have been involved in the degradation of these peptides. The fact that tissue factor could hydrolyze the His^9Leu^{10} bond only slowly is consistent with perfusion studies with [10-*Leu*-^{14}C]-angiotensin I which indicated no hydrolysis of this bond by rat lung in one study (19) and only 3% hydrolysis in another study (23). In this regard, it might be noted that tissue factor could contribute to "angiotensin-converting enzyme" activity in crude tissue preparations when activity is measured by the rate of release of His-Leu from angiotensin I.

Tissue factor extensively degraded substance P. Boileau *et al.* (29) reported that substance P was inactivated 12% upon a single passage through the pulmonary circulation of the rat. Since angiotensin-convert-me would not be expected to hydro- stance P because of its C-terminal ond (30), the observed disappear- this peptide in lung may be due to sis by tissue factor.

previously suggested (4) that tissue nay be similar or identical to other -bound peptidases which have been in other organs (31-36). Indeed, ry of the ubiquitous nature of tissue 22) suggests that this enzyme may be partially responsible for the destruc- vasoactive peptides reported for sev- fused vascular beds other than lung) and for homogenate and particu- ctions from a variety of tissues (27, 45).

it evidence has suggested that tissue ay be available to hydrolyze circu- eptides without at the same time g blood coagulation. Maynard *et al.* ve shown that tissue factor exists on side of plasma membranes of cul- ISH amnion cells and human skin sts. Yet, tissue factor coagulant ac- masked, possibly by the cell surface hus, it is possible that circulating could penetrate the surface coat of endothelial cells and interact with ctor, whereas the large protein mol- actor VII, could not. Another possi- based on the suggestion by Smith an (47) that enzymes which hydro- culating peptides may be located caveolae intracellulares which are the vascular lumen but covered with te diaphragm, as is the case for 5'- dase. If tissue factor is located hese structures, it is possible that the gm may be permeable to peptides ermeable to circulating factor VII er clotting factors. Disruption of the gm during tissue damage may be ry to expose tissue factor and initiate tion.

resent investigators eliminated the ity that tissue factor used in these is contaminated with pancreatic-type peptidase B; catheptic carboxypep- 3, and plasma carboxypeptidase N nases) since these enzymes would be d to release the C-terminal arginine man fibrinopeptide A (48). The fact ue factor is active at neutral pH (4), is sensitive to thiol-reducing agents, but is not affected by DFP, soybean trypsin inhibitor or Ca^{2+} (4) suggests that this preparation is not contaminated by some of the several known kininases or angiotensinases (27, 48). Furthermore, there is no effect of Cl$^-$ on tissue factor up to 0.1 *M* using Leu-Gly-Gly as substrate, suggesting that the preparation does not contain angiotensin-converting enzyme (28). Clotting and peptidase activity, however, remain coincident for the extensively purified relipidated enzyme (4).

The ability of tissue factor to degrade physiologically active peptides suggests important functions for this enzyme in addition to those related to blood coagulation. Recognition of the full significance of tissue factor peptidase activity *in vivo* may depend on the discovery of a highly specific inhibitor which will enable investigators to detect participation of tissue factor in various physiological mechanisms.

Summary. Tissue factor apoprotein and relipidated tissue factor preparations extensively hydrolyze bradykinin, Lys-bradykinin, Met-Lys-bradykinin, substance P, [Asp^1,Ile^5]-angiotensin II, [Asp^1,Ile^5]-angiotensin I, and human fibrinopeptide A while acting more slowly on [Sar^1,Ile^5]-angiotensin II, [Me$_2$Gly^1,Ile^5]-angiotensin II, bradykinin potentiating pentapeptide from *B. jararaca*, luteinizing hormone-releasing hormone, melanocyte stimulating hormone-release-inhibiting factor (Pro-Leu-Gly-NH$_2$), and oxytocin. No hydrolysis of thyrotropin-releasing factor or bradykinin potentiating nonapeptide from *B. jararaca* is observed. Relipidated and apoprotein tissue factor act at identical rates under the conditions of the assay. Dansylation and chromatography of tissue factor–peptide incubation mixtures further indicate that relipidated and apoprotein tissue factor also hydrolyze peptides by identical mechanisms. No fewer than six bonds are hydrolyzed in bradykinin while the angiotensins and substance P are degraded to constituent amino acids. Only the N-terminal alanine is released from fibrinopeptide A. 2-Mercaptoethanol greatly inhibits the hydrolysis of bradykinin by relipidated tissue factor.

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ogenic Properties of a Nonproducer Malignant Tumor Induced by Murine Sarcoma Virus (39445)

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: sarcoma virus (Moloney) M- MuLV) and other pseudotypes of arcoma virus induce tumors in in- lt mice that invariably regress. mors have been inappropriately d rhabdomyosarcomas or sarco- The major portion of the tumorous ing following intramuscular or sub- s injection of virus in mice is a nass ("atypical granuloma") which ed contain varying proportions of t mesenchymal foci (2). Through f some of these tumors in immunodeficient mice it has been possible malignant populations of cells that progressively and kill intact synge- ients. We have reported the selec- development of hemangiosarcomas passage in thymectomized C57BL/ Hf/LW F_1 ($BC3HF_1$) mice of tu- luced originally by M-MSV(M- 3). The hemangiosarcoma desig- *M-1* (4) did not produce infectious murine leukemia virus (MuLV), tive for *gs* antigen, but contained a MSV genome rescuable by -MuLV (R-MuLV) and also con- ccasional C-type particles. Thus, ntal cell line resembled the 3T3 cell lines induced *in vitro* by M- . *XM-1* was found to contain virus- transplantation rejection antigens 4).

derivatives were obtained *in vitro* le cell isolates of neoplasm *XM-1* lant generation 47 (6). Several ere characterized as to their virus n, presence of *gs* antigen, growth istics, and immunogenicity and im- sitivity in tumor rejection. In con- e parental *in vivo* passaged *XM-1* , none of the clones contained res- MSV. Low levels of infectious ere detected in some clones, but the presence or absence of MuLV did not influence immunogenicity or immunosensitivity of these cloned tumors as detected by virus-specific tumor rejection (6).

One of these clonally derived cell lines, *H-11* (HP) was of particular interest. Infectious virus was not detectable, nor was virus inducible with BrdU. Preliminary data suggested that *H-11* (HP) was highly immunogenic in *in vivo* tumor rejection experiments and tumor growth was inhibited in M-MSV-immunized syngeneic $BC3HF_1$ recipients. We now report further experiments that we believe distinguish between virus-specific, new cell surface antigens (TSTA) and virion antigens in this nonproducer cell line.

Materials and methods. *H-11* (HP) cells were grown continuously in Eagle's minimal essential medium with 10% fetal bovine serum (GIBCO) and antibiotics (100 U of penicillin and 100 μg of streptomycin/ml). *H-11* (HP) cells were periodically assayed in syngeneic mice for their tumor-producing capacity. After several passages, progressively growing tumors were produced only with large inocula in the range of 5–10 × 10^6 cells/mouse. Several *in vitro* passages of *H-11* (HP) examined have been negative for infectious virus by XC and MSV focus forming assay. By [^{3}H]uridine labeling and sucrose gradient centrifugation techniques there was no evidence of virus particles with a density of 1.16 g/cm^3 detected in the supernatant, nor was virus associated with RNA-dependent DNA polymerase detected. MSV was not recoverable by cocultivation with MuLV-carrier fibroblasts or superinfection with high-titered M-MuLV or R-MuLV. Neither bromo- nor iododeoxyuridine was capable of inducing infectious virus. MuLV group specific antigens (*gs*) were also not detected in any of the several assays (CF and immunofluorescence). Radioimmunoassay indicates the presence of

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gp 70 (13 to 30 ng/10^6 cells). The significance of gp 70 in tumor rejection, however, has not been adequately assessed. It would appear therefore that these nonproducer *H-11* (HP) cells contain, in all likelihood, a strikingly repressed, integrated MSV or MuLV genome. Loss of a potentially integrated viral genome does not appear likely.

Syngeneic (BC3H)F_1 mice were immunized using several regimens of subcutaneously inoculated *H-11* (HP) cells that were trypsinized, washed, and resuspended in serum-free medium. Specificity of tumor rejection was assayed through immunizations with M-MSV(M-MuLV) and with the polyoma virus-induced sarcoma #89 adapted to *in vitro* growth. Because of the low oncogenic potential of the continuously passaged *H-11* (HP) clonal line, tumor challenge was made with *H-11* passaged *in vivo* and then grown in tissue culture (*H-11* short passage). These cells were then aliquoted and frozen. The TD_{50} of this standardized preparation was 1 × 10^4 cells.

Results and discussion. It may be seen from the results of four separate experiments (Figs. 1A and B and Tables I and II) that immunization with nonproducer *H-11* (HP) cells provided protection in syngeneic hosts against grafted *H-11* tumor cells. This protection was evident in the frequency of progressively growing tumors as well as in

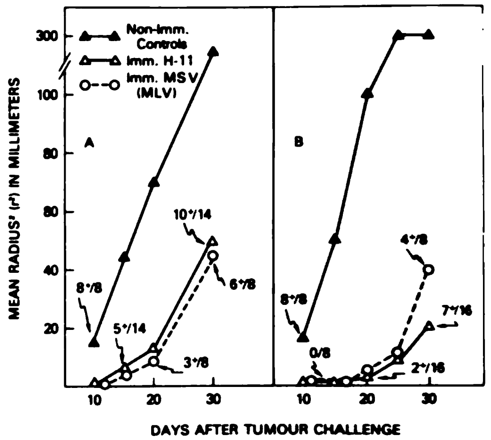

FIG. 1. Syngeneic (BL × C3H)F_1 female mice, 12 to 16 weeks of age, in both groups A and B, were immunized with 2 × 10^6 nonproducer *H-11* (HP) cells subcutaneously, once a week ×5, or with M-MSV, 1 ml subcutaneously, once a week ×3. Virus concentration = 5 × 10^5 focus-forming units (ffu)/ml. All groups were challenged 10 days after last immunization with 1 × 10^5 *H-11* cells. All mice in the A group received 300 R whole body X irradiation 24 hr prior to tumor challenge. (▲——▲), nontreated controls; (△——△), immunized with *H-11* (HP) cells; (O- - -O), immunized with M-MSV. Numbers 8+/8; 10+/14, etc. = fraction of mice with progressively growing tumors. Days of observation = 70; frequencies of tumors did not change beyond 30 days. Mean age to death of controls in group B = 32.6 days; mean age to death of *H-11* immunized mice = 70.1 days. *H-11* (HP) immunized mice also challenged with tumor #89 a polyoma virus-induced sarcoma. See text.

TABLE I. IMMUNOLOGIC SPECIFICITY OF IMMUNIZATION WITH NON-PRODUCER *H-11* (HP) CELLS AND POLYOMA VIRUS.

	Immunization[a]		
Challenge tumor	*H-11* (HP)	Polyoma virus or #89 cells (fraction of mice with progressive tumors at 70 days)	None
H-11 (5 × 10^4 cells)	1/6[b] (17%)	12/17[c] (70%)	10/12 (83%)
#89 (1.5 × 10^5 cells)	5/6 (83%)	4/17[d] (24%)	10/12 (83%)

[a] (BC3H)F_1 female mice, 12–16 weeks of age, immunized as follows: *H-11* (HP) cells at 1–2 × 10^6 cells once a week ×5; polyoma virus (LID-1 strain) inoculated weekly ×3 at a concentration of 5 × 10^6 plaque-forming units per mouse; tumor #89 cells inoculated into footpad and amputation done at 2 weeks. Tumor challenges were done at 2 weeks after the last immunization.

[b] Difference in frequency = $0.5 > p > 0.01$ as compared with the combined data obtained with the other (polyoma immunized and nonimmunized control).

[c] Inhibition of tumor growth also reflected in rate of growth of *H-11* tumor; thus, mean age to death of controls = 40 days and of *H-11* immunized mice = 75 days.

[d] $p < 0.01$ as compared with the combined data obtained with the other two groups (H-11(HP) immunized and nonimmunized control).

BLE II. IMMUNOLOGIC SPECIFICITY OF IMMUNIZATION WITH NONPRODUCER *H-11* (HP) CELLS.

tu-		Fraction of mice with progressive tumors (mean radius[2]) at day					Mean age to death (days)
		15	20	25	35	50	
	Immunized[a]	0/8	2/8(2.3)	6/8(9.5)	6/8(50)	–	46
s)	Nonimmunized	6/8(20)	7/8(64.5)	7/8(>100)	7/8(>100)	–	35
	Immunized[a]	2/8	2/8	2/8	7/8	8/8	70
s)	Nonimmunized	2/8	2/8	2/8	5/8	7/8	70

H)F$_1$ female mice, 12–16 weeks old, immunized with *H-11* (HP) tissue culture-passaged cells at 2 ×
× 5; challenged with tumors *H-11* and #89 10 days after last immunization.

of growth, measured as mean ra-
mean days to death of tumorous
reimmunization with the homolo-
is strain M-MSV(M-MuLV) also in-
esistance to challenge with *H-11*.
sults along with those reported ear-
oup cross-reactivity among several
ultured from *XM-1* tumor clearly
the existence on *H-11* (HP) cells of
cific transplantation antigens. Irra-
300 R, whole body) of *H-11* (HP)
ed mice before challenge did not
heir capacity to reject *H-11* synge-
or cells (see Fig. 1A).
pecific nature of the immune re-
nduced by nonproducer *H-11* (HP)
en in the results detailed in Tables I
he polyoma virus-induced sarcoma
has its own virus-specific TSTA (7)
gressively in *H-11* (HP) immunized
c mice but was rejected in mice
ed with polyoma virus or #89 cells.
immunization with polyoma virus
ells did not induce rejection of *H-*

ear, therefore, from these data that
nsformed cells (in this study using a
ne of malignant hemangiosarcoma
ned *in vivo*) do contain MSV-spe-
face antigens capable of evoking
ntation immunity. Since no evi-
virus production or of viral finger-
as found by any of the available
our cloned *H-11* (HP) tumor line,
ntigens or surface antigens associ-
n virus production are not responsi-
umor rejection. MSV-induced new
ntigens, analogous to the TSTA of
rus-induced neoplasms, appear to
nsible.
vey-MSV-induced neoplasm of the
D$_4$ hamster and an M-MSV(M-MuLV)-induced neoplasm of the rat both reported to be "nonproducers" were shown previously (8, 9) to possess tumor-associated antigens of the transplantation type; in both cases, however, MSV could be rescued by "helper" viruses and both contained *gs* antigen. An MSV-associated antigen on the surface of MSV-transformed nonproducer cells was demonstrated by immunoelectron microscopy (10). However, its relationship with TSTA is not known. Others have found no evidence for the existence of distinctive surface antigens on nonproducer MSV-induced neoplasms using serologic (11, 12) or transplantation rejection (13) assays.

Although a complete MSV genome has not been detected, it cannot be ruled out that a protein of the viral genome exists in *H-11* (HP) cells that codes for the TSTA. Experiments are in progress to detect a portion of the virally related genome by molecular hybridization.

Summary. A cloned cell line *H-11* (HP) derived from an MSV-induced neoplasm (a hemangiosarcoma) was found to possess virus-specific tumor rejection antigen(s). The specific nature of the immune response was established through the use of a polyoma virus-induced neoplasm #89 both in cross immunization and cross challenge experiments. Virus antigens or surface antigens associated with virus production are not responsible for tumor rejection since virus production could not be detected, nor were any viral fingerprints of MSV found by the several assays used.

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s of Experimental Diabetes on Intestinal Strontium Absorption in the Rat[1]
(39446)

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lkaline earth element, strontium, to the same family as magnesium ium. All are better absorbed in the than in the distal small intestine d show enhancement of transport in D (2, 4, 5). Calcium and magne- e important nutrients, whereas n is of interest as a contaminant of water in the form of its radioactive . Strontium behaves much like cal- its transport (1, 2). Duodenal cal- nsport is depressed in the rat with or streptozotocin induced diabetes ut magnesium transport is the same trol rats (8). These effects are pres- when duodenal mucosal growth is d in diabetic as compared with con- . We therefore examined strontium t and effects of diabetes.

als and methods. Male Simonsen its (Simonsen Labs, Gilroy, Calif.) nercial rat chow (Wayne Lab Blox, 1.2%; magnesium, 0.2%; phospho- %; Allied Mills, Libertyville, Ill.) d. Rats to be made diabetic were single intraperitoneal injection of repared streptozotocin solution (50 citrate buffer at pH 4.5, 110 mg/ ight-matched controls received cit- ffer only. Diabetic rats studied glycosuria greater than 2% (Tes- li Lilly Co.) and fasting blood sugar 0 mg% (Dextrostix, Ames Co.).

ium absorption was measured 5 or after injection in animals fasted t but receiving water *ad libitum.* re anesthetized with sodium pento- (Nembutal, 40 mg/kg) and placed n a warm heating pad. The abdo- opened by a midline incision and mon bile duct ligated. Cannulas were inserted and tied in place so that duodenum and terminal ileum (15–25 cm proximal to the cecum) could be perfused intraluminally. The perfusion solution contained strontium chloride (2.5 mM), sodium chloride (165 mM), and nonabsorbed indicator: polyethylene glycol (PEG, 1.0 g/liter), and ^{14}C-PEG (0.6 mCi/g, 0.01 mCi/liter, New England Nuclear, Boston, Mass.). Using a syringe, each segment was flushed with 5–10 ml of normal saline solution followed by an air flush. A 10-ml volume of test solution contained in a plastic centrifuge tube was recirculated through each segment at a constant rate of 2 ml/min (Bowman Pump, Process and Instruments, Brooklyn, N.Y.). To facilitate mixing, the intake tubing from the pump rested at the bottom of the reservoir and the return tubing emptied near the top. During the *in vivo* perfusion, the abdomen was closed, kept moist with saline-dampened gauze, and rectal temperature was maintained at 35–38°. After 2 hr, the test solution was collected in the reservoir and blood was immediately drawn from the aorta for determination of glucose concentrations. Duodenum, terminal ileum, and the intervening midgut were excised, residual contents evacuated by gently pressing the segment flat on absorbent paper, and length and wet weight of segments were measured. To obtain mucosa, each segment was slit lengthwise, spread on a glass plate with mucosa up, and scraped horizontally with a microscope slide (nonbeveled). Tissue was placed in a preweighed crucible, weighed, dried in a vacuum oven at 100° for 18–20 hr, and reweighed to obtain dry weight.

Strontium concentrations were measured by atomic absorption spectrophotometry (Perkin-Elmer, Model 303, Norwalk, Conn.) in aliquots of the initial perfusion solution and final reservoir contents. Liquid

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scintillation counting was used to count ^{14}C-PEG: 0.2 ml of sample, 3.8 ml of water, 1 drop of 4% stannous chloride, and 15 ml of Bray's scintillation fluid. Plastic or acid washed (1 N HCl) glassware was used throughout.

Net transfer rates for strontium were calculated as follows:

$$\text{Net transfer } (\mu\text{moles/hr}) = \frac{V[C_i - (C_f \times {}^{14}\text{C-PEGR})]}{\text{hr}}$$

where V is initial volume (liter) of solution recirculated, C_i and C_f are the initial and final concentrations of strontium (μM) in the luminal solution, ^{14}C-PEGR is the ratio of initial to final ^{14}C-PEG concentration, and hr is hours recirculated. Net transfer was divided by both mucosal weight (g) and length of segment (cm). Results are expressed as mean values ± one standard error. The Student's t test for unpaired data was used for statistical comparisons, and probability values less than 0.05 were considered significant.

Results and discussion. Table I gives mean data on body weight and small intestinal measurements for control (C) and diabetic (D) rats studied 5 and 12 days after injection. The number of rats used in the study for each group is given at the top of the table and applies to succeeding tables. Mean body weight at time of injection (Initial), just prior to overnight fasting before study (Final), and at time of perfusion (Fasted) are shown at the top of Table I. Body weights did not differ between groups initially. Control rats gained 5-6 g/day in contrast to diabetics who failed to gain weight.

Mean total length and weight of the small intestine was greater in diabetics at both time intervals, but the increase was significant at 12 days only. Weight of small intestine as a percentage of fasted body weight was greater in diabetics at both 5 and 12 days. At 12 days, the percentage for diabetics (4.7%) was double that of controls (2.3%). Total mucosal dry weight in diabetics was 13% greater than in controls at 5 days and 50% greater at 12 days, in contrast to no difference in underlying tissue. Hence, intestinal growth stimulation in diabetic animals is localized primarily to the mucosa.

Mucosal mass expressed as dry weight (mg/cm) is given at the bottom of Table I for the three divisions of the total small intestine. Mucosal mass per centimeter tended to decline progressively from duodenum to ileum for all groups. Mucosal dry weight (mg/cm) of duodenum and midgut was significantly greater in diabetics at both 5 and 12 days, whereas in the ileum, mucosal dry weight was greater only at 12 days.

TABLE I. BODY WEIGHT AND INTESTINAL MEASUREMENTS FOR CONTROL AND DIABETIC RATS.

	Mean data			
	5 days		12 days	
	Control (N = 8)	Diabetic (N = 8)	Control (N = 10)	Diabetic (N = 9)
Body weight (g)				
Initial	201	207	200	211
Final	227	196**	269	197**
Fasted	209	175**	246	167**
Total small intestine length (cm)	94	99	94	108*
Total small intestine weight (mg)	5323	5864	5562	7802**
(Weight of small intestine/body weight) × 100	2.5	3.4**	2.3	4.7**
Dry weight (mg)				
Total mucosa	729	822	738	1101**
Total underlying tissue	422	404	518	528
Mucosal dry weight (mg/cm)				
Duodenum	8.1	9.7*	7.8	11.6**
Midgut	8.0	8.4*	8.0	9.9*
Ileum	6.8	6.4	7.4	9.7**

[*] Diabetic data differ significantly from control data, $p < 0.05$.

[**] Diabetic data differ significantly from control data, $p < 0.001$.

TABLE II. Strontium Absorption for Control and Diabetic Groups.

	5 days		12 days	
	Control	Diabetic	Control	Diabetic
	(µmoles/hr, mean ± SE)			
per cm × 100				
duodenum	57 ± 3	40 ± 4*	55 ± 4	41 ± 3*
ileum	18 ± 1	16 ± 3	16 ± 2	12 ± 2
per g dry wt mucosa				
duodenum	74 ± 4	44 ± 5**	77 ± 6	37 ± 2**
ileum	28 ± 3	27 ± 5	23 ± 3	13 ± 2*

* Diabetic data differ significantly from control data, $p < 0.05$.

** Diabetic data differ significantly from control data, $p < 0.001$.

Strontium absorption data for duodenum and ileum of both control and diabetic groups at 5 and 12 days are shown in Table II. Data are expressed per centimeter of segment length and per gram of dry weight mucosa. Absorption per centimeter of length defines absorptive capacity of the segment and was lower in diabetics in duodenum at both time intervals. Ileal absorption per centimeter was about one-third that of duodenum for both groups. Although mean ileal absorption per centimeter was slightly lower in diabetics, differences from control values were not significant. Absorption expressed per gram of dry weight of mucosa defines absorptive specific activity of the segment and shows the same relationships as per unit length. Duodenal absorption per gram of mucosa of diabetics was half that of controls. Ileal absorption was much less than duodenal for both groups. Ileal absorption by diabetics differed from controls only at 12 days.

Net luminal water movements in perfused segments were minimal (not shown). In duodenum, mean values ranged from 2% net absorption to 8% net secretion. In ileum, mean values ranged from 0 to 6% net absorption.

The pattern of absorption of strontium in duodenum and ileum of control and diabetic rats (Table II) is similar to that previously observed for calcium (6, 7): duodenal transport is depressed. Ileal transport is less affected, but there is depression of specific absorption in diabetics at 12 days. Thus, diabetes affects strontium and calcium absorption in the same manner. Effects of diabetes on calcium absorption appear to be mediated by abnormal vitamin D metabolism: (a) depressed duodenal calcium absorption in the diabetic rat is restored to the level of controls by 1,25-dihydroxycholecalciferol, the vitamin D metabolite that acts directly on the intestinal mucosa (9); (b) duodenal calcium absorption in the diabetic rats is also corrected by extract of the plant, *Solanum malacoxylon* (10). *Solanum malacoxylon* extract, like 1,25-dihydroxycholecalciferol, also acts directly on the mucosa to stimulate calcium absorption. Since vitamin D shows similar effects on transport of calcium and strontium (2), these findings on strontium absorption in diabetes fit with a defect in vitamin D metabolism and are another example of parallelism between calcium and strontium transport.

Summary. Control and streptozotocin diabetic rats were studied at 5 and 12 days after induction of diabetes. Strontium absorption was measured by *in situ* perfusion of duodenum and ileum. Duodenal absorptive capacity (absorption per unit length) and absorptive specific activity (absorption per gram of dry weight mucosa) were depressed. Depression was present both at 5 days, when mucosal growth is similar in controls and diabetics, and at 12 days, when mucosal growth is 50% greater in diabetics. Effects of diabetes on ileal absorption were minimal in comparison with effects on duodenum. This depression of duodenal strontium absorption in the diabetic rat is analogous to effects of diabetes on calcium absorption and may be mediated by abnormal vitamin D metabolism.

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NGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 593–597 (1976)

Kinetics of the Rapid Action of Interferon (39447)

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was recently found that treatment of (under physiological conditions) with eron for only a few minutes renders resistant to virus challenge (presum- due to the "antiviral protein"). Thus, lefense mechanism can act more rap- han does the spread of virus in infected s. The purposes of the present study to examine (a) some of the variables lead to early-appearing resistance in n cell cultures and (b) some molecular s during this rapid induction of resist-

terials and methods. Human leukocyte eron and human fibroblast interferon obtained from the Antiviral Sub- es Program, NIAID, and were pro- l by methods previously described (3, he interferon-induced antiviral activity easured by the inhibition of the yield dbis virus hemagglutinin in a single- yield assay (12). Interferon titers are as reference units per milliliter. Un- therwise specified, experiments were cted in tube cultures of human fore- fibroblasts incubated in a 37° water Culture medium and diluent was Ea- medium containing 2% fetal bovine and antibiotics. At the end of each ation period, the interferon was re- d and the cultures washed four times Earle's balanced salt solution before nge with Sindbis virus to determine ral activity. All media and wash fluids prewarmed to 37° and kept warm ghout the experiment.

ults. Effect of temperature on the de- nent and decay of the antiviral state. In rst series of experiments, interferon at temperature was applied to the human in fibroblast cultures (strain HFS-1). transfer to the incubator it required 40 to 60 min for the temperature to rise to 37° as determined by an electronic thermometer. As shown in Fig. 1A, application of 5 units/ml of interferon before placing the tubes in the incubator resulted in the initiation of viral resistance between 2 and 4 hr, with the resistance becoming maximum at 6 hr. Thereafter, resistance remained relatively stable as long as the interferon remained in the extracellular fluid (1). Figure 1B shows the effect on established antiviral activity of removal of interferon as compared with its retention. Removal of interferon was preceded by an overnight incubation with interferon to allow the prior development of full antiviral activity. It may be seen that the level of antiviral activity dropped drastically between 10 and 15.5 hr after removal of interferon. In comparison, the replacement of interferon resulted in maintenance of antiviral activity although the level of resistance manifested moderate fluctuation which is characteristic of this system (1; Buckler, C. E., and Baron, S., personal communication, 1970). In most strains of human foreskin cells (including the HR203 strain which was used in the rapid action experiments below) maximum resistance was reached at 6 hr using 3 to 10 units/ml of interferon. However, under the same conditions other human fibroblasts developed maximum resistance at 17 to 20 hr, i.e., the MA 308 human nasal polyp cells (Microbiological Associates) and the continuous line, human U cells (from Dr. K. Cantell).

Similar types of experiments were performed again in human foreskin fibroblast cultures (strain HR203) but with careful temperature maintenance at 37° throughout the experiment. Figure 2 shows the development and decay of antiviral activity after exposure of these cells to 10, 30, or 300 units of human interferon for 5 min before

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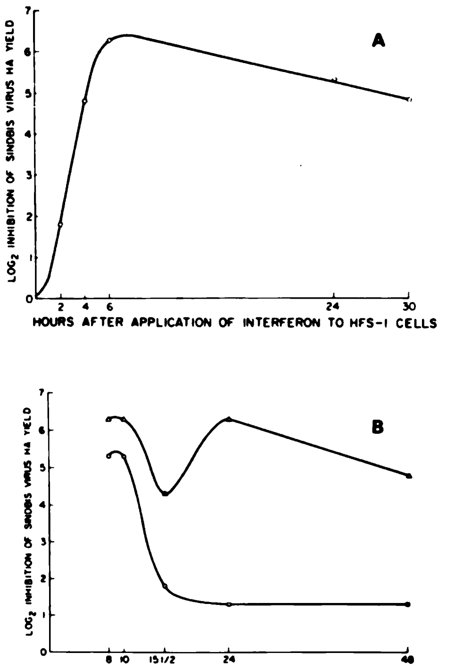

FIG. 1. Development (A) and decay (B) of the antiviral state in human foreskin cultures treated with 5 units/ml of interferon. The interferon was applied to cultures at room temperature before immediate incubation at 37°. In (B) interferon was removed and replaced with medium (O——O) or removed and replaced with the same concentration of interferon (Δ——Δ).

removal and washing. It may be seen that substantial resistance to virus challenge was present at the time interferon was removed (5 min) and that the degree of resistance continued to rise so that between 1 and 8 hr after reaction with interferon resistance was above the measurable level.

In the cultures treated with 10 units of interferon, decrease of resistance was measurable between 8 and 12 hr after removal of interferon and resistance was no longer detectable by the 16th hr. In the cultures treated with 30 or 300 units/ml of interferon, the onset of decreased resistance was delayed until 12 hr after removal of interferon and resistance was similarly delayed.

These findings extend the observation that rapid induction of resistance is influenced by the temperature of incubation and the concentration of interferon (5). Specifically, application of 10 or more units/ml of human interferon to human cells, held constantly at 37°, causes rapid development of resistance to virus challenge. The effects of high concentrations of interferon at temperatures below 37° are being studied. The decay kinetics of the established resistance appear to be approximately the same whether the resistance was induced under conditions favoring rapid or slow development (compare Fig. 1B to Fig. 2).

For the experiments below, treatment with 30–300 units/ml of interferon for 1 to 30 min, at 37°, was considered to represent conditions for rapid induction. The level of resistance which develops between 1 and 30 min of treatment is essentially the same (5).

Effect of prior chilling of cells on subsequent development of resistance. It seemed possible that application of cold or room temperature medium to cells might derange cellular metabolism for a prolonged time after rewarming of the cells. This could account for the slower development of resistance by cells exposed to interferon initially at room temperature and subsequently incubated (Fig. 1A) as compared with continuous incubation (Fig. 2). Therefore, development of resistance in cells previously chilled and then warmed was compared to that of unchilled cells. The human cell cultures were initially exposed to ice bath temperatures for 30 min, then warmed to 37° in a waterbath and exposed to 100 units/ml of interferon for 4 min before Sindbis virus

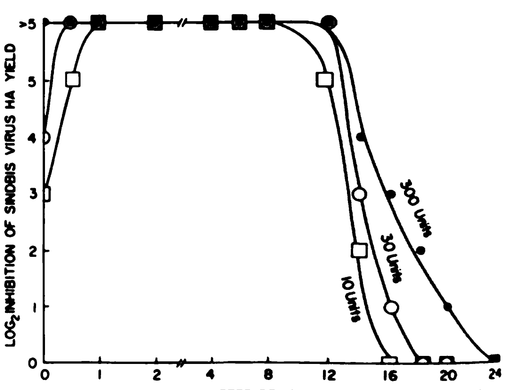

FIG. 2. Development and decay of the antiviral state in human foreskin cultures treated with 10, 30, or 300 units/ml of interferon for 5 min. Interferon, media, and cultures were maintained at a constant 37°.

nge. The level of resistance (5 $\log_2$ tion of yield) induced under this con- of prior chilling was equal to that of ɔl cultures constantly maintained at ;his finding mitigates against the possi- that chilling of cells results in pro- d impairment of the ability of cells to nd to interferon.

ısible priming action of the applied in- ɔn. It is possible that the interferon ;d for a brief time primes the cells so the challenge virus induces a more and more effective interferon re- e (priming) which then inhibits further :eplication (8). To test this possibility, ınce induced by challenge virus was ed by actinomycin D. Specifically, the ultures at 37° were exposed to 300 ml of interferon for 20 min, washed :imes, incubated at 37° for 1 or 2 hr, ıen challenged with Sindbis virus in the nce or absence of 10 μg/ml of actino- ı D. This dose of actinomycin D in- d 95% of cellular RNA synthesis ı 10 min without affecting virus syn- . As shown in Table I, actinomycin D h was shown to prevent completely the tion of interferon or the antiviral re- ce by challenge virus) did not signifi- ʳ change the degree of resistance as ared with cultures challenged in the ce of actinomycin D. This finding indi- that the brief exposure of cells to inter- did not function to prime the cells to quent production of the antiviral state ɛ challenge virus.

E I. Development of Resistance after ɪɪɴ Exposure to Interferon under Condi- s Preventing Interferon Induction by ʟʟenge Virus.

: of chal- after re- l of inter- ›n (min)	Actinomycin D present during viral adsorption	Yield of Sindbis virus HA ($\log_2$)
ɜated with :feron	No	5
ɜated with :feron	Yes	5
0	No	1
60	No	<1
60	Yes	1
120	No	<1
120	Yes	<1

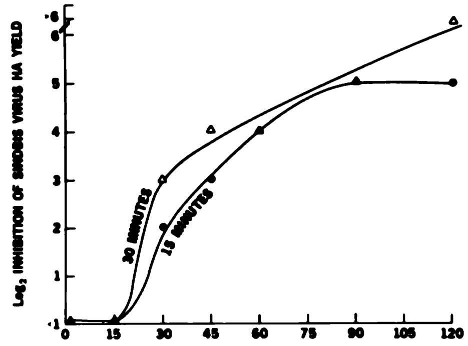

Fig. 3. Actinomycin D timing of the production of the mRNA for the antiviral protein. Cultures were treated with 300 units/ml of interferon at 37° for 15 (○——○) or 30 (△——△) min before washing and incubation for the indicated times before applying 10 μg of actinomycin D and challenging with virus 2 hr later.

ıetics of the actinomycin D effect. The inhibition of viral growth in cultures challenged immediately after a 5-min exposure to interferon does not necessarily mean that the factors responsible for resistance (antiviral protein) are produced by 5 min. Resistance probably develops prior to 4 hr after exposure to interferon since (a) to inhibit virus, resistance must occur before virus replication is complete (6–7) hr for many viruses) and (b) interferon may be effective when applied as late as 2.5 hr after infection (10, 14). To establish this time more closely, the time of production of the mRNA for the resistance factors was determined since it was shown previously that the mRNA is rapidly translated (4). Sets of cultures were treated with 300 units/ml of interferon for 15 or 30 min at a constant 37°. After washing with warm medium, cultures were incubated for varying times before applying 10 μg/ml of actinomycin D. The cultures were then incubated for an additional 2 hr before virus challenge so that any delayed translation of mRNA would have been completed. Since this dose of actinomycin D rapidly and irreversibly inhibits 95% of mRNA production, the development of resistance plotted against the time of application of actinomycin D approximates the kinetics of production of the mRNA for the antiviral protein. It may be seen in Fig. 3 that the addition of actinomycin D, up to 15 min after removal of inter-

TABLE II. EFFECT OF INHIBITON OF PROTEIN SYNTHESIS ON INDUCTION OF THE mRNA FOR THE ANTIVIRAL PROTEIN.

Treatment before addition of actinomycin D	Yield of sindbis virus HA ($\log_2$)	Reduction of sindbis virus HA ($\log_2$)
None	6	–[a]
Cycloheximide	6	0
Interferon	1	5
Interferon and cycloheximide	1	5

[a] Not applicable.

feron, completely inhibited the development of the antiviral state. When actinomycin D was added between 15 min and 2 hr after removal of interferon, there was a gradual development of resistance. Since the interferon had been applied initially for 15 to 30 min and since production of mRNA did not begin for an additional 15 to 30 min, it may be concluded that the first production of the mRNA began between 30 and 45 min after the application of interferon. This requirement for RNA synthesis suggests that the antiviral protein did not exist in a masked form prior to the application of interferon but rather was newly, as well as rapidly synthesized.

Lack of requirement of protein synthesis for transcription of the mRNA for the antiviral protein. It was previously demonstrated (under conditions of slower induction of the antiviral state) that the mRNA for the antiviral protein was directly induced by interferon and did not require the production of a new protein for this derepression (4). This demonstration was based on the production of the mRNA in the absence of protein synthesis and its subsequent translation into the antiviral protein in the presence of actinomycin D. To help determine whether rapid induction of the mRNA for the antiviral protein also did not require the prior induction of a new protein, the cells were treated with 100 units/ml of interferon for 2 hr in the presence or absence of an inhibitor of protein synthesis, cycloheximide (10 μg/ml). Protein synthesis was inhibited by 92%. Cycloheximide inhibition was then reversed by washing the cultures with warm medium, and 10 μg/ml of actinomycin D was added before virus challenge. Since mRNA for the antiviral protein may be produced in the presence of cycloheximide but not later in the presence of actinomycin D, any development of resistance (antiviral protein) would indicate direct induction of the mRNA for the antiviral protein without a requirement for a new, intermediary protein. As shown in Table II, full resistance does develop in the presence of the inhibitor treatment, confirming the lack of requirement for a new, intermediary protein.

Discussion. The results presented here indicate that the rapid development of resistance following interferon treatment was not observed previously (1) for two reasons. First, holding interferon-treated cultures at 4° or at room temperature delays up to 60 min the time at which cell cultures reach incubator temperature for normal metabolic activity. Second, the earlier experiments were performed with levels of interferon below those required for rapid induction. The present findings extend the observation that 10 or more units/ml of interferon rapidly induce resistance. Thus, under conditions of temperature and interferon concentration which mimic those *in vivo* (5), the antiviral action of interferon is extremely rapid.

The kinetics and the molecular events involved in rapid induction of resistance appear to be qualitatively similar to those occurring during slow induction of resistance but the rate is increased. Thus, after brief exposure to interferon, the mRNA for the antiviral protein first appears between 30 and 45 min. If translation of the human mRNA is as rapid as in the mouse system, then the antiviral protein probably is produced shortly thereafter (4). It is noteworthy that, although substantial resistance occurs after very brief exposure to interferon, maximum antiviral activity does not occur until 2 to 4 hr (5).

Studies are underway to help determine whether rapid development of resistance results from the previously observed, cell-bound interferon (2, 6, 9, 11, 13).

The present experimental conditions provide a time period during genetic derepression when the inducer may no longer be present and the induction has not yet occurred. This possibility is being tested. Study of the cellular events between inducer reaction with the cell and the derepression of the cistron might be possible in this system.

Summary. The present study was undertaken to examine some factors which lead to early-appearing resistance in human cells treated with interferon. It was previously shown that two conditions required for rapid development of resistance were continuous maintenance of cultures at 37° and use of more than 10 units/ml of interferon. The decay kinetics of the established resistance appear to be approximately the same whether the resistance was induced under conditions favoring rapid or slow development. With the use of actinomycin D it was shown that the mRNA for the antiviral protein is produced between 30 and 45 min after the first contact with interferon. Ruled out were the possibilities that a priming action of interferon and a newly synthesized intermediary protein were necessary for rapid development of resistance.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 152, 598–602 (1976)

Effect of Human Nasal Secretions on the Antiviral Activity of Human Fibroblast and Leukocyte Interferon[1] (39448)

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Since the discovery of interferon (IF), investigators have sought to utilize this antiviral agent clinically. One potential use of IF would be in treating or preventing viral respiratory tract infections, since it has been implicated in recovery from such infections (3). In addition, the antigenic diversity of viral pathogens complicates control by conventional vaccination approaches.

Most clinical trials with intranasally applied IF have failed to show efficacy (8). However, Merigan *et al.* recently demonstrated that clinical efficacy against a rhinovirus challenge was achieved when large quantities of human leukocyte IF were administered (10).

Because several reports have indicated that various body fluids are capable of inhibiting IF (4, 5, 14), we examined human nasal secretions for the presence of an inhibitor that could account for the apparent large dose requirement necessary for clinical efficacy. The effect of human nasal secretions on the antiviral activity of both human fibroblast and leukocyte IF was investigated because of the described antigenic and biochemical differences between leukocyte and fibroblast IF (1, 15).

Materials and methods. Nasal secretions. Nasal secretions from healthy volunteers were collected and concentrated 10-fold by a previously described procedure (13). Total protein concentrations were determined by the technique of Lowry *et al.* (9).

Interferon preparations. Both human fibroblast and leukocyte IF were provided by the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases. Fibroblast IF was prepared by Havell and Vilček (7) using human foreskin fibroblasts and polyriboinosinic:polyribocytidylic acid as the inducer. Leukocyte IF was prepared from buffy coat leukocytes stimulated with Sendai virus and purified according to the technique of Cantell (2).

Cell cultures. Human foreskin fibroblasts (cell strain HR-202) from HEM Research Inc. (Rockville, Md.) were used to assay IF. Mouse L-cells were used for plaque assays of vesicular stomatitis virus (VSV). Both cell lines were propagated in Eagle's minimum essential medium with Earle's salts (MEM) purchased from Grand Island Biological Co. (Grand Island, N.Y.). MEM was supplemented with 10% heat inactivated fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml), and *l*-glutamine (10 m*M*). Cells were cultured at 37° in an atmosphere of 5% CO_2 and 95% air.

Interferon assay. A yield reduction assay was used to measure IF activity. Samples for assay were diluted in MEM containing 5% FBS and 1-ml aliquots were added in duplicate to HR-202 cell monolayers (1.0×10^6 cells) in 10 × 35-mm plastic petri dishes (Lux Scientific Corp.). After incubation at 37° for 18–24 hr, the medium was removed and the cells were washed once with phosphate-buffered saline (PBS) deficient in calcium and magnesium ions (6). The HR-202 monolayers were then challenged with VSV at a multiplicity of infection of at least 10. After adsorption for 1 hr at 37°, the inoculum was removed and the cells were washed four times with PBS. The monolayers were then refed with 2 ml of MEM containing 2% FBS and incubated at 37°. After 24 hr, the fluid was collected and centrifuged at 1400*g* for 10 min, and the supernatant fluid was frozen at −70° until assayed for VSV.

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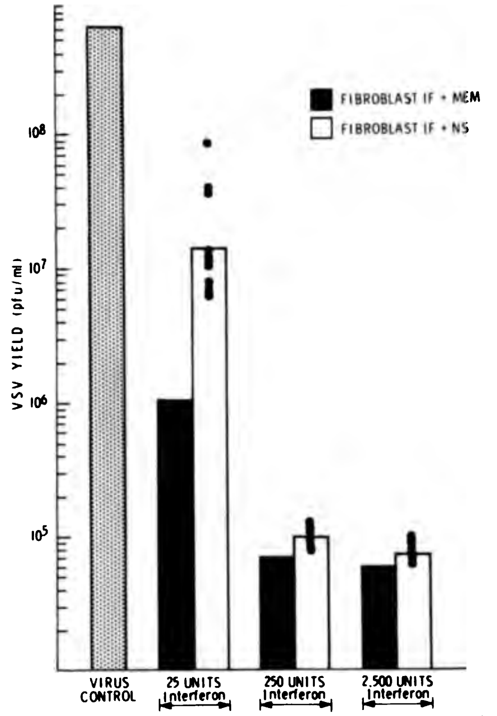

FIG. 1. Effect of human nasal secretions on the antiviral activity of human fibroblast interferon (IF). The results following incubation of 10 individual nasal secretions (NS) with 25, 250, or 2500 units of IF are represented by dots; geometric means are represented by open bars.

Virus yields were determined by plaque assay in L-cells. One unit of IF was defined as that dilution of the original sample which reduced the VSV yield by 50% compared to controls. In this system, 5 units of IF were equivalent to 1 unit of the human IF research standard 69/19 from the Medical Research Council (Mill Hill, London, England). An internal standard was included with most assays and the titer did not vary more than twofold.

Results. Effect of nasal secretions on varying doses of human fibroblast IF. To determine the effect of nasal secretions on fibroblast IF, 0.15 ml of concentrated nasal secretions from 10 individuals was each mixed well with an equal volume of MEM (without serum) containing 75, 750, or 7500 units of fibroblast IF. As controls, the same quantities of IF were mixed with MEM. All mixtures were incubated for 4 hr at 34°, to simulate the temperature of the upper respiratory tract. The volume of each sample was then increased to 3.0 ml to yield final IF concentrations of 25, 250, or 2500 units/ml, and 1.0-ml aliquots were assayed for IF activity. Twenty-five units of fibroblast IF incubated with MEM reduced the virus yield from 7.0×10^8 plaque forming units (PFU)/ml (control) to 1.1×10^6 PFU/ml (Fig. 1). However, when the same amount of IF was incubated with nasal secretions from 10 individuals, an equivalent reduction in virus yield did not occur. Yields of VSV varied from 6.6×10^6 to 9.4×10^7 PFU/ml, representing a 6- to 85-fold increase in virus yield and a loss of 38 to 91% of the IF activity. The mean virus yield from cells treated with IF and nasal secretions was increased by more than 2 standard deviations over the IF control virus yield. The loss of IF activity was attributable to interaction with nasal secretions, since nasal secretions alone in this assay system increased the virus yield by an average of only 1.7-fold. The inhibition of IF activity was overcome when the same nasal secretions were incubated with 250 or 2500 units of fibroblast IF (Fig. 1).

Comparison of the effect of nasal secretions on human fibroblast and leukocyte IF. Six nasal secretion specimens were incubated with approximately 25 units of human fibroblast or leukocyte IF (Fig. 2). Three of the nasal secretions incubated with fibroblast IF did not significantly increase virus yields and therefore did not inhibit IF. However, three other nasal secretions did increase virus yields by 13- to 18-fold. In this experiment the mean increase in virus yield was also 2 standard deviations greater than the IF control. In contrast to the results obtained with fibroblast IF, five of these six nasal secretions did not significantly enhance virus yields when incubated with leukocyte IF.

Similar experiments have been repeated with a total of 28 nasal secretions from different individuals. Using a fourfold increase in virus yield as evidence of inhibition of IF activity, 57.1% of the nasal secretions tested inhibited fibroblast IF, whereas only 10.7% of the same nasal secretions inhibited leukocyte IF (Table I). Moreover, a 4.6-fold increase in virus yield was the greatest increase noted with leukocyte IF,

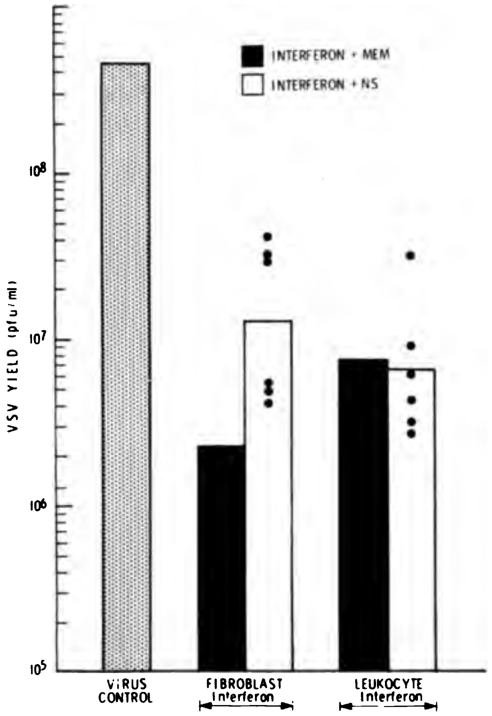

FIG. 2. Effect of human nasal secretions on the antiviral activity of human fibroblast and leukocyte interferon. The results following incubation of six individual nasal secretions (NS) with approximately 25 units of fibroblast or leukocyte interferon are represented by dots; geometric means are represented by open bars.

whereas nasal secretions showing inhibitory activity toward fibroblast IF caused an average virus yield increase of 15.7-fold.

To rule out the possibility that leukocyte IF was simply less sensitive than fibroblast IF to the inhibitor in nasal secretions, increasing amounts of nasal secretions were incubated with fibroblast or leukocyte IF. To conduct this and subsequent experiments, a nasal secretion pool was prepared by combining the secretions obtained from 25 volunteers.

Fifty units of fibroblast or leukocyte IF in a constant volume (0.15 ml) were incubated with 0.075, 0.15, 0.3, or 0.6 ml of the nasal secretion pool. This gave a ratio (volume of nasal secretion to volume of IF) of 0.5:1, 1:1, 2:1, or 4:1, respectively. Neither IF preparation was noticeably affected by incubation with increasing volumes of MEM (Fig. 3). In addition, the increase in nasal secretion volume did not result in a concomitant increase in virus production in the absence of IF. However, when increasing volumes of the nasal secretion pool were incubated with fibroblast IF, a linear increase in virus yield (decrease in IF activity) was noted; a 4:1 volume ratio of nasal secretion to IF resulted in total inhibition of 50 units of fibroblast IF. In contrast, leukocyte IF was not inhibited by increasing amounts of nasal secretions.

Preliminary characterization of the IF inhibitor(s) in nasal secretions. In three sepa-

TABLE I. NUMBER OF NASAL SECRETIONS (NS) SHOWING INHIBITION OF INTERFERON ACTIVITY.

Experiment	Number of NS	Number of nasal secretions showing inhibitory activity against[a]	
		Fibroblast IF	Leukocyte IF
1	6	6	0
2	6	3	1
3	7	3	1
4	7	3	1
5	2	1	0
Totals	28	16 (57.1%)	3 (10.7%)

[a] Defined as an increase in virus yield of fourfold or greater compared to the interferon control.

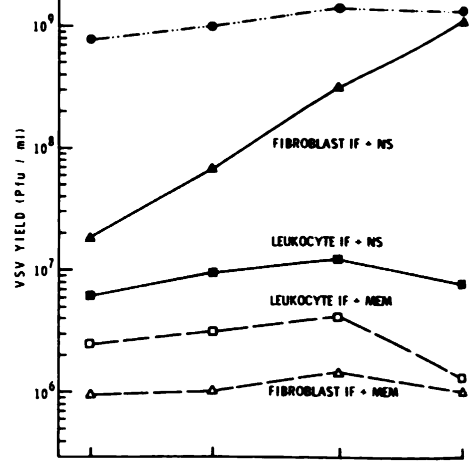

FIG. 3. Effect of increasing volumes of nasal secretions (NS) on the antiviral activity of human fibroblast and leukocyte interferon (IF).

experiments, the inhibitory activity was
test in those nasal secretions with the
est total protein determinations. Initial
npts to identify this inhibitor in nasal
tions were carried out by incubating
blast IF with pure preparations of three
e major proteins [i.e., albumin, lyso-
e and IgA (11, 12)] found in nasal se-
ons. A range of physiological concen-
ons of albumin (0.1, 1.0, and 10.0 mg/
Behring Diagnostics), lysozyme (0.1,
and 10.0 mg/ml, Miles Laboratories),
7S IgA (0.15, 1.5, and 15.0 mg/ml,
ring Diagnostics) did not possess inhibi-
activity. Therefore, to characterize the
erties of the inhibitor in nasal secre-
, portions of the nasal secretion pool
subjected to the following treatments
then tested for inhibitory activity. Nasal
tions were dialyzed for 3 hr against
changes of 400 ml of PBS. The inhibi-
vas nondialyzable. Heating at 56° for 1
d not deplete nasal secretions of inhibi-
activity. Portions of the nasal secretion
were incubated at 37° for 1 hr with
in (5 mg/ml, Flow Laboratories, Inc.).
reaction was stopped by the addition of
at a final concentration of 33%. The
itor was not destroyed and the pres-
of the increased serum concentration
ot appear to influence the IF assay.

e nasal secretion pool was also mixed
inuously with 10 vol of ether for 5 min.
aqueous phase was dialyzed against
and tested for inhibitory activity. Fol-
g this treatment, the nasal secretion
did not inhibit IF. Portions of the nasal
tion pool were subjected to 100,000g
hr in a Beckman Model L3-50 ultra-
ifuge using a Type 40 rotor. After cen-
gation, the top two-thirds of the fluid
recentrifuged at 100,000g for 2 hr. The
itory activity was found in the upper
thirds of the fluid following the second
rifugation.

scussion. We have examined human
l secretions for the presence of an inhib-
of human fibroblast or leukocyte IF.
presence of such an inhibitor might ex-
the need for such large doses of leuko-
IF to show *in vivo* efficacy (10). The
iral activity of small quantities of hu-
fibroblast IF was partially inhibited when mixed with human nasal secretions. This inhibition was overcome with increased concentrations of fibroblast IF. Human leukocyte IF, however, was not inhibited by human nasal secretions.

In view of the described antigenic (1) and biochemical (15) differences between fibroblast and leukocyte IF, it is not surprising that biological differences, such as sensitivity to an inhibitor, do exist. Other body fluids which have been shown to inhibit fibroblast IF are serum (4, 14), saliva, cerebrospinal fluid, bile, an extract of feces (4), and urine (5). The same body fluids were not tested against leukocyte IF. However, an inhibitor of leukocyte IF in serum has been demonstrated (Stanton and May, personal communication).

Nasal secretions are a complex mixture of substances which include at least 12 of the proteins found in plasma (12) as well as six components which cannot be detected in plasma (11). Although our results do not allow a definitive identification of the inhibitor, three of the major proteins intrinsic to nasal secretions (i.e., albumin, lysozyme, and 7S IgA) do not appear to be responsible. The characteristics of the inhibitor found in nasal secretions resemble those of an inhibitor found in fetal bovine and human serum (14). These investigators suggested that the inhibitor they described might be associated with a lipoprotein. Our results concerning the properties of the inhibitor described in this report are not inconsistent with a similar interpretation.

Although an inhibitor of human fibroblast IF was found in the present study, it does not explain the results obtained in clinical trials with human leukocyte IF, since no inactivation or inhibition of leukocyte IF by human nasal secretions could be demonstrated. The inhibitor of fibroblast IF, however, could be of significance if this preparation were to be considered for clinical use.

Summary. Many normal human nasal secretions contain an inhibitor of human fibroblast IF. This inhibitor had no effect on human leukocyte IF. The amount of inhibition of fibroblast IF increased with increasing quantities of nasal secretions. Also, the inhibition could be overcome with increasing concentrations of IF.

We would like to thank Shellie Scott and Christopher Thomas for their excellent technical assistnace.

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neous Development of Gluconeogenesis in Fetal Rat Livers during Incubation in Organ Culture (39449)

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neogenesis, one of the most impor- ctions of the rat liver, develops rap- r birth but remains dormant in the -3). It is not clear whether this is the enzymatic machinery, though is suppressed or because *de novo* s of gluconeogenic enzymes is initi- y after birth. Much of the available tion has been accumulated with fe- anipulated while being retained in us (4, 5). Investigations with fetal *in situ* are, however, subject to ncontrollable variables which make tation of some of the data uncertain. of the inherent limitations of work- intact fetuses, we have chosen to tal rat liver in organ culture in the ion that this system, being more rig- defined and easier to control, would a model for studies in the develop- perinatal gluconeogenesis (6).

lier experiments with fetal rat liver , we found that gluconeogenesis mine spontaneously developed with ring incubation. Our results corre- ll with the observation of Mandelli) and Dodek *et al.* (8) that phospho- vate carboxykinase (EC 4.1.1.32,) of liver spontaneously increased organ culture. These findings are nt with the conclusion that PEPCK iting enzyme in the regulation of genesis in the perinatal rat (9). e energy needs of the newborn ani- probably met largely by lipid, the ment in the liver of alternate gluco- pathways may be expected. In this e present evidence that the livers of ld fetal rats utilize glycerol and ser- ot alanine and lactate as substrates oneogenesis. Fetal livers in organ onvert all substrates to glucose or .

Materials and methods. Fetuses from anesthetized CFE rats (Carworth Farms, New City, N.Y.) were taken surgically under sterile conditions at 19 to 21 days of gestational age as determined from their weight and crown-rump length (10). Liver fragments were established in culture as described by Wicks (11). The medium was Eagle's minimum essential medium with Hanks' balanced salt solution containing 0.11 *M* glucose and 0.008 *M* sodium bicarbonate (12). Incubation was at 37° under a mixture of 98% air-2% carbon dioxide. Measurement of incorporation of radioactive precursors into glucose and glycogen are as described in a previous paper (13). Explants directly incubated with the radioactive compounds are called "fresh" livers; those incubated in medium for 42 hr, then transferred to fresh medium containing radioactive precursors are called "aged" livers.

Results. In Table I, the incorporation of radiotracer from L-[U-^{14}C]-alanine, L-[U-^{14}C]serine, L-[U-^{14}C]lactate or D-[U-^{14}C]glycerol into glucose and glycogen of freshly isolated and "aged" fetal liver are compared. In "fresh" livers there was almost no incorporation of label from [U-^{14}C]alanine or [U-^{14}C]lactate into glucose or glycogen. Incorporation of label into carbohydrate residues increased significantly after 42 hr of preincubation, in confirmation of our previous results. [^{14}C]alanine and [^{14}C]lactate were about equally incorporated into free glucose. Although [^{14}C]alanine was a somewhat better precursor of glycogen than was [^{14}C]lactate, their overall utilization for gluconeogenesis was not significantly different because glycogen comprised only 5 to 10% of the carbohydrate formed.

[^{14}C]Serine was incorporated into both

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rved.

TABLE I. INCORPORATION OF RADIOACTIVE PRECURSORS INTO GLUCOSE AND GLYCOGEN BY FETAL RAT LIVER EXPLANTS IN ORGAN CULTURE.[a]

Substrate	Replicates	Preincubation (hr)	Glycogen (mg/g wet weight)	^{14}C in glycogen (dpm/dish)	^{14}C in glucose (dpm/dish)	^{14}C in total carbohydrate (% of dose)
L-[U-^{14}C]alanine	4	0	13.81 ± 4.25	43 ± 3	0	0.004 ± 0.0005[b]
	7	42	0.79 ± 0.42	250 ± 153	3890 ± 1630	0.36 ± 0.16
L-[U-^{14}C]lactate	4	0	16.1 ± 5.7	104 ± 9	142 ± 115	0.019 ± 0.008[b]
	4	42	0.93 ± 0.10	101 ± 27	3100 ± 520	0.25 ± 0.14
L-[U-^{14}C]serine	5	0	23.8 ± 1.4	228 ± 104	3249 ± 239	0.16 ± 0.001[b]
	6	42	0.39 ± 0.07	1060 ± 204	11060 ± 1042	0.62 ± 0.05
D-[U-^{14}C]glycerol	4	0	17.6 ± 5.1	3470 ± 975	21825 ± 805	1.08 ± 0.08
	4	42	0.50 ± 0.12	1887 ± 628	40490 ± 7650	1.80 ± 0.35

[a] Explants were from animals 20 to 21 days of gestational age. Activities added to culture dishes were 0.5 μCi [U-^{14}C]alanine (sp act., 140 Ci/mole); 0.5 μCi of [U-^{14}C]glycerol (sp act, 112 Ci/mole); 0.5 μCi of [U-^{14}C]lactate (sp act, 137 Ci/mole); 1 μCi of [U-^{14}C]serine (sp act, 153 Ci/mole). Final concentration of substrate was 3.4 mM. Values shown are means ± standard error.

[b] $P < 0.005$ for comparison of incorporation of label into preincubated and freshly isolated liver.

glycogen and glucose in fresh livers. The total incorporation was increased about fourfold in aged livers. In the case of [U-^{14}C]glycerol, incorporation of label into total carbohydrate was initially high in fresh liver. Preincubation did not significantly increase gluconeogenesis or the relative distribution of ^{14}C from glycerol into glycogen and glucose. Under all conditions, gluconeogenesis from glycerol was higher than from any of the other labeled molecules studied. It will be noted that preincubation resulted in extensive loss of glycogen from the explants. The remaining glycogen was of high specific activity. Thus, while glycolysis greatly exceeded glycogenesis from glucose, gluconeogenesis appeared to be the predominant mode of glycogen synthesis.

Discussion. We have found, in accord with the observations of others (1–3), that gluconeogenesis does not take place in rat liver during fetal development. However, gluconeogenesis from alanine or lactate is initiated after liver explants are maintained in organ culture for an interval of 42 hr. During this preliminary incubation period important changes occur, as indicated by the increased response of the tissue to steroid hormones (11). The nature of these changes is unknown but may involve in part the destruction of inhibitory hormones and other molecules. It is important to note that, even after preliminary incubation, the rate of gluconeogenesis is much slower in explants than in the intact postnatal rat. Where incorporation of 3-carbon precursors is measured in minutes in the latter, significant incorporation requires hours in the former. The machinery of gluconeogenesis is therefore present in the fetal liver but in a state of rudimentary development.

The behavior of serine and glycerol as gluconeogenic substrates in fresh fetal liver is consistent with the conclusion that the route including phosphoenolpyruvate plays a controlling role in the development of gluconeogenesis from pyruvate, lactate, or alanine. Serine is transformed into glucose in two possible ways: through serine dehydratase to pyruvate or through L-serine:pyruvate aminotransferase to glycerate. The latter route bypasses the pyruvate-phosphoenolpyruvate sequence (15–17). In fresh liver, this pathway predominates; in aged liver, the phosphoenolpyruvate carboxykinase-dependent pathway emerges to provide an additional port of entry, and consequently there is a rise in incorporation of serine into carbohydrate. Recent evidence indicates that hydroxyproline, like serine, is an important gluconeogenic precursor after birth (15). Enhanced utilization of hydroxyproline involves conversion of one of its metabolites through serine to phosphoglycerate and thence to glucose.

In contrast with the effects of incubation on the incorporation of serine into glucose and glycogen, incorporation of glycerol is initially high in fresh liver and is increased by less than twofold after preincubation. This is because glycerol enters the gluconeogenic pathway at the triose phosphate level through glycerol kinase which is known to be active in fetal liver (18-20) and totally bypasses phosphoenolpyruvate carboxykinase.

The ability of the fetal liver to utilize glycerol more efficiently than other potential substrates may reflect the postnatal requirements. The energy needs of the newborn are probably met largely from lipid. Among the physiological changes consistent with increased lipid metabolism after birth are a rise in plasma free fatty acid, a fall in respiratory quotient from 1.0 to 0.7, and an increase in the level of ketone bodies and glycerol in the plasma. Therefore, glycerol, resulting from hydrolysis of storage and dietary lipid, may be an important substrate for gluconeogenesis (21, 22).

The preexisting enzymes allow the animal to utilize lipid rapidly as a valuable alternative source of glucose. The evidence, therefore, suggests that the appearance of gluconeogenesis after birth is dependent on both the net synthesis of enzymes and the initiation of the expression of preexisting enzymes.

Summary. Freshly isolated fetal liver explants in organ culture did not convert L-[^{14}C]alanine or L-[^{14}C]lactate to carbohydrate, but L-[^{14}C]serine and D-[^{14}C]glycerol were both transformed. When explants were subjected to 42 hr of preliminary incubation without supplements, followed by transfer to fresh medium with added precursor, all four substrates underwent gluconeogenic transformation. It was concluded that the ability of fetal rat liver in organ culture to convert alanine and lactate to carbohydrate evolves slowly, but the conversion of glycerol, and to a lesser extent serine, to glucose and glycogen is initiated immediately

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Lutropin Receptors from Male and Female Tissues: Different Responses to a Lutropin Receptor Binding Inhibitor (39450)

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We recently reported a lutropin receptor binding inhibitor (LH-RBI) which strongly inhibits the binding of ^{125}I-labeled ovine lutropin ([^{125}I]oLH) to its ovarian receptor site (1, 2). The LH-RBI was found in pregnant and pseudopregnant rat ovary, but was not found in either the ovary of nonpregnant mature rats and immature rats or in extra-ovarian tissues such as liver, oviduct, and testis. The LH-RBI was estimated to have a molecular weight of approximately 3800 as determined by Sephadex G-50 column chromatography and was heat stable and also protease sensitive. It had been shown by us (1) that LH-RBI did not appear to be due to endogenous lysosomal enzyme activities (20-min boiling of the LH-RBI did not destroy its inhibitory activity); also, there was no detectable quantity of [^{125}I]oLH bound to the LH-RBI (chromatographic data). In our present work, we investigate the effect of the LH-RBI on the binding of [^{125}I]oLH to testicular LH receptors. The results show that LH-RBI does not inhibit LH binding to testicular LH receptors, although the inhibition is highly significant in the ovary. Possible implications of the inhibition pattern for the mechanism through which the LH-RBI exerts its binding-inhibitory effect are discussed in the present paper.

Materials and methods. Ovine lutropin was prepared as described previously (3). Lutropin was labeled with ^{125}I using the chloramine-T method as described previously (4). Pseudopregnancy in the female Sprague–Dawley rat was induced at the age of 25 days by single subcutaneous injection with 50 IU of pregnant mare serum gonadotropin, followed 65 hr later by single subcutaneous injection of 25 IU of human chorionic gonadotropin (hCG) as described by Parlow (5). Five days after the injection of hCG, ovaries were obtained from rats sacrificed with CO_2 inhalation. The organs were trimmed free of fat and immediately frozen on dry ice. The frozen ovaries were stored at −20° until use.

The ovarian and testicular receptors were prepared as follows: Pseudopregnant ovaries were homogenized in 6 vol of Solution A (0.1 *M* Tris buffer, pH 7.4, containing 5m*M* $MgCl_2$ and 0.1 *M* Sucrose, plus 0.1% bovine serum albumin). The homogenization was done with a glass–Teflon tissue homogenizer with a loose-fitting pestle driven by motor. The homogenization was accomplished by 10 strokes exactly. Testes from adult male rats were decapsulated, weighed, and homogenized in 3 vol of Solution A. Homogenization was done in a Duall-type hand homogenizer with loose-fitting pestle using exactly 20 strokes. The ovarian and testicular homogenates were strained through two thin layers of cheesecloth, and filtrates separately centrifuged at 5000*g* for 15 min at 4°. The ovarian supernatant was centrifuged again at 30,000*g* for 15 min, and the 30,000*g* supernatant was used as the ovarian extract which contained the LH-RBI. The ovarian pellets were washed three times by centrifugation with 8 ml of Solution A per wash, resuspended in Solution A, and used as the ovarian receptor preparations for the incubation studies. In previous studies (6), it was found that three washes were adequate to remove the LH-RBI in the receptor preparation. Since the LH-RBI activity was not present in the rat testes (1), the testicular pellets were washed only once, resuspended in Solution A, and used as the testicular receptor preparation for the incubation studies.

In a standard binding assay, an aliquot (0.1 ml) of the 5000*g* pellets equivalent to 10 mg of ovary or testis, 0.1 ml of the ovarian extract (LH-RBI), and 10 μl containing 1 ng of [^{125}I]oLH were added in sequence in each tube (Falcon plastic tubes.

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5 mm). In some of the tubes, 10 μg) of unlabeled oLH was added to s the control to determine the non- binding of [^{125}I]oLH. The final vol- each tube was 0.22 ml. The tubes ıcubated for 1 hr at 37°, chilled, and iately centrifuged at 12,000g for 10 4°. The supernatants were withdrawn pellets were washed once with 0.5 Solution A. The tubes were again ıged and the pellets obtained were l in a well-type scintillation gamma (Nuclear-Chicago, Model 4224). ta from all of the LH binding experi- lescribed in this paper represent the binding of [^{125}I]oLH to the ovarian icular receptors. Specific binding is l as the total binding of [^{125}I]oLH he nonspecific binding of [^{125}I]oLH. cific binding is the uptake of labeled ıe in the presence of 1000-fold ex- unlabeled hormone and is usually 8- f the total binding. This amount of cific binding was not further reducidditional wash with Solution A. The ance, if any, of the nonspecific bindınknown. Each assay point was acıied by a parallel nonspecific binding ination.

lts and discussion. Evidence that LH- es not inhibit labeled lutropin bind- varian lutropin receptors by a prefer- inding of the hormone to the inhibi- tead of the receptor) or by a selective of lutropin by LH-RBI is summarized in Table I, Experiment I. The [^{125}I]-oLH was first incubated with the LH-RBI for 1 hr at 37° before incubation with the ovarian LH receptors. The results indicate that preincubation of labeled lutropin with LH-RBI did not lead to greater inhibition of binding, a result consistent with earlier studies (1) indicating LH-RBI was not attributable to either degradative enzymes or soluble lutropin binding components (e.g., soluble receptors). Therefore, the inhibition seems to be due to one of two mechanisms: (i) competition between the LH-RBI and [^{125}I]oLH for the same binding site on the receptor; (ii) binding of LH-RBI and [^{125}I]oLH to different sites on the receptor, with the binding of the LH-RBI at the second site preventing the binding of [^{125}I]oLH at the lutropin binding site.

The effect of the LH-RBI on the binding of lutropin to the hormone receptors of ovary or testis was next compared. This comparison is summarized in Fig. 1. In view of the experimental results indicating that the LH-RBI does not inhibit the binding of [^{125}I]oLH to testicular LH receptors (Fig. 1), the possibility of the competition between the LH-RBI and [^{125}I]oLH for the same binding site becomes unlikely (*vide supra,* mechanism (i)). If the LH-RBI inhibited the [^{125}I]oLh binding through the mechanism of competition for the same binding site, the LH-RBI would have inhibited the binding of [^{125}I]oLH to the testicular LH receptors. By elimination of al-

E I. EFFECTS OF INCUBATION SEQUENCE ON THE INHIBITION OF [^{125}I]oLH BINDING BY LH-RBI TO OVARIAN OR TESTICULAR LH RECEPTORS.[a]

Incubation systems: Preincubation mixture[b]	Incubation systems: Additives after preincubation	Specific binding of [^{125}I]oLH (%)	Inhibition of binding (%)
[^{125}I]oLH + Solution A[a]	Ovarian receptors	23.89	Control
[^{125}I]oLH + LH-RBI	Ovarian receptors	9.85	59
[^{125}I]oLH	LH-RBI + ovarian receptors	10.15	58
Ovarian receptors + Solution A	[^{125}I]oLH	15.50	Control
Ovarian receptors + LH-RBI	[^{125}I]oLH	1.95	88
Ovarian receptors	LH-RBI + [^{125}I]oLH	5.58	64
Testicular receptors + Solution A	[^{125}I]oLH	9.66	Control
Testicular receptors + LH-RBI	[^{125}I]oLH	9.58	1
Testicular receptors	LH-RBI + [^{125}I]oLH	9.65	0

letails see text.

preincubation and the final incubation were carried out at 37° for 1 hr as described in Methods.

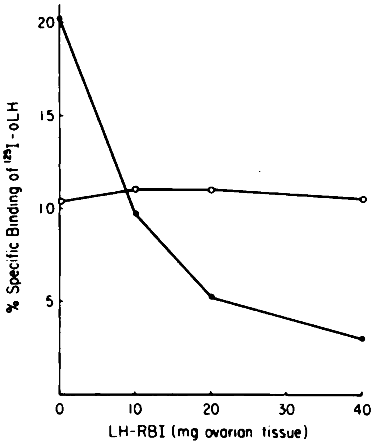

FIG. 1. Different effects of the LH-RBI on the specific binding of [^{125}I]oLH to the 5000g pellets (receptor preparations) of testicular (O-O-O) and ovarian (●—●—●) homogenates. The pellets were incubated with [^{125}I]oLH in the presence of various concentrations of the LH-RBI (pseudopregnant ovary extract, see methods section). LH-RBI concentration is expressed as milligram equivalents of ovarian tissue. The incubation was carried out at 37° for 1 hr as described in the methods section.

ternatives, the data thus favors mechanism (ii) (*vide supra*). One may argue that the failure of the LH-RBI to show the inhibition in the testicular system could also be due to low binding affinity of the LH-RBI for the LH binding site in the testis. We examined this possibility using two criteria: (i) preincubating the testicular receptors with the LH-RBI for 1 hr at 37° before the addition of [^{125}I]oLH and a second incubation (see Table I, Experiment III) and (ii) increasing the quantity of the LH-RBI for the incubation from 10 mg tissue equivalent up to 40 mg tissue equivalent (Fig. 1). Again, however, no inhibition of the binding of [^{125}I]oLH to testicular LH receptors was observed in either of these two conditions (Table I and Fig. 1). Experiment II, Table I, was included to compare ovarian responses to lutropin and LH-RBI effects on ovarian tissue under the same incubation sequences applied to the testicular tissue (Experiment III). Although the inhibition difference for the sequence of addition (Experiment II, 88 vs 64%) could be interpreted by either mechanism (i) or (ii) (*vide supra*), these data, when considered with the data in Fig. 1 and Experiment III, Table I, are only consistent with mechanism (ii).

Our suggestion that the LH-RBI inhibits the LH binding through the mechanism other than competitive inhibition was also supported by the results of the binding kinetics study. The ovarian LH receptors were incubated with increased concentrations of [^{125}I]oLH in the absence or presence of a fixed concentration of the LH-RBI. The results were used to construct the Scatchard plot (7) in Fig. 2. The dissociation constant, i.e., K_d for the [^{125}I]oLH binding, was essentially the same in either the absence or presence of the LH-RBI. Since the association constant (the reciprocal of K_d) of the [^{125}I]oLH binding was not reduced by the presence of the inhibitor, this would suggest that the LH-RBI did not compete with [^{125}I]oLH for the same binding site on the receptor. On the other hand, the displacement of the curve obtained in the presence of the LH-RBI indicated the total number of available LH binding sites is significantly reduced (i.e., the x-axis intercept is proportional to the number of available LH binding sites).

The phenomenon of "allosteric" or "different site" inhibition as applied to the enzyme-substrate-inhibitor interaction has

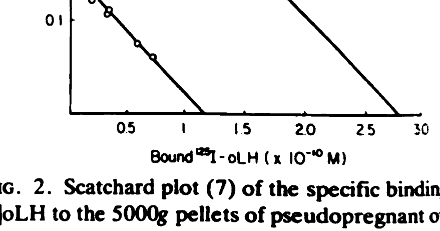

FIG. 2. Scatchard plot (7) of the specific binding of [^{125}I]oLH to the 5000g pellets of pseudopregnant ovarian homogenates in the presence (O-O-O) or absence (●-●-●) of the LH-RBI. The slope is equivalent to 1/ K_d; the K_d was calculated to be 7.56×10^{-10} M (O-O-O) or 7.52×10^{-10} M (●-●-●). Molecular weight of ovine LH was taken as 30,000.

been recognized in the field of enzymology. A similar control mechanism is also likely to occur in hormone–receptor interactions.

The present work suggests that the lutropin receptor of ovaries, but not of testes, has a specific LH-RBI binding site in addition to the lutropin binding site; and that the binding of the LH-RBI produced an "allosteric" type of inhibition to the binding of lutropin at the lutropin binding site. In this respect, a demonstration of the specific binding of the LH-RBI to the ovarian lutropin receptor would serve as direct evidence for supporting the existence of such a phenomenon. Since a preparation of a homogeneous LH-RBI material suitable for radioactive labeling has not been achieved in our laboratory, we are not able, at this stage, to provide the direct evidence clarifying this possibility.

Summary. An inhibitor for lutropin receptor site binding (LH-RBI), which strongly inhibited the binding of ^{125}I-labeled ovine lutropin ([^{125}I]oLH) to ovarian LH receptors, did not inhibit the [^{125}I]oLH binding to testicular LH receptors. Preincubation of the LH-RBI with [^{125}I]oLH did not affect the binding of preincubated [^{125}I]oLH to ovarian LH receptors. No inhibition of [^{125}I]oLH binding to testicular LH receptors was observed even when the concentration of LH-RBI was significantly increased or when the testicular LH receptors were first incubated with LH-RBI prior to the addition of [^{125}I]oLH and a second incubation. Scatchard analysis revealed that the dissociation constant of [^{125}I]oLH binding was essentially the same in the presence or absence of LH-RBI. The results suggest that: (i) the lutropin receptor of ovaries, but not of testes, has a specific LH-RBI binding site in addition to the lutropin binding site, and (ii) the binding of the LH-RBI produces an "allosteric" type of inhibition to the binding of lutropin at the lutropin binding site.

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Environmental Effects on Glutathione-Insulin Transhydrogenase in Rat Liver[1] (39451)

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Glutathione-insulin transhydrogenase (GIT, thiol-protein disulfide oxidoreductase, EC 1.8.4.2) catalyzes an interchange of sulfhydryl and disulfide groups on proteins and small molecules. In practice, the enzyme catalyzes the reduction of the disulfide bridges of insulin in the presence of excess glutathione (1, 2). *In vitro,* this reduction of insulin is accompanied by the oxidation of the sulfhydryl groups of glutathione. Since it has been shown by Chandler and Varandani (3) that other sulfhydryl-containing proteins may replace glutathione in the reaction, the actual substrate pair utilized *in vivo* is not clear at this time.

The GIT-catalyzed reduction of insulin has been shown to be the rate-controlling step in insulin degradation. This raises the possibility that GIT may function to mediate insulin action (4). Glutathione-insulin transhydrogenase is widely distributed in a number of organs in several species (5). Measurements of the specific activity of GIT in various tissues have shown that those most sensitive to insulin action, i.e., heart, skeletal muscle, and fat, have the lowest levels of GIT, while insulin-insensitive tissues such as the liver and kidney exhibit the highest levels of this enzyme (6). More recently glutathione-insulin transhydrogenase has been shown to be inducible by insulin injection in diabetic rats (7).

The studies described in this paper were designed to investigate several aspects of the regulation of GIT under various dietary, developmental, and hormonal conditions. In order to relate GIT activity to the amount of enzyme present in the material analyzed, the assay for the enzyme described by Chandler and Varandani (8) utilizing ^{125}I-labeled insulin was modified accordingly.

Methods. Albino male rats were obtained from the Holtzman Rat Company, Madison, Wisconsin. For the developmental experiments pregnant rats of known gestation were used. The mothers were allowed access to a 25% protein diet *ad libitum.* The newborn rats were separated from their mothers just prior to sacrifice. Livers from the same littermates were collected and pooled. For the metabolic-regulation experiments rats were housed in a regulated light-dark room, lighted from 8:00 AM to 8:00 PM. They were allowed access to semipurified diets containing 0, 12.5, 60, or 90% protein from 8:00 AM to 4:45 PM for 10 days and sacrificed between 7:00 and 8:00 AM. For the glucagon experiments rats were allowed access to a 25% protein diet *ad libitum* for 10 days. Glucagon (Eli Lilly) was injected subcutaneously at doses of 1.0 mg/100 g body weight. Controls were injected with the diluting solution (1.6% glycine and 0.2% phenol). For the cortisone experiments rats were adrenalectomized 3 days prior to injection. They were allowed access to a 25% protein diet and water containing 0.9% NaCl *ad libitum.* Cortisone (Pfizer Corp.) was injected intraperitoneally at doses of 10 mg/100 g body weight. The animals were sacrificed at the times indicated in the results.

The rats were sacrificed by decapitation. The liver was quickly removed and homogenized in 4 vol of 0.44 *M* sucrose with 10 strokes of a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 12,000*g* (SS-34 rotor, Sorvall centrifuge, 8500 rpm) for 10 min. The supernatant was centrifuged at 105,000*g* (50 Ti rotor, L2-65B Beckman ultracentrifuge, 41,000 rpm)

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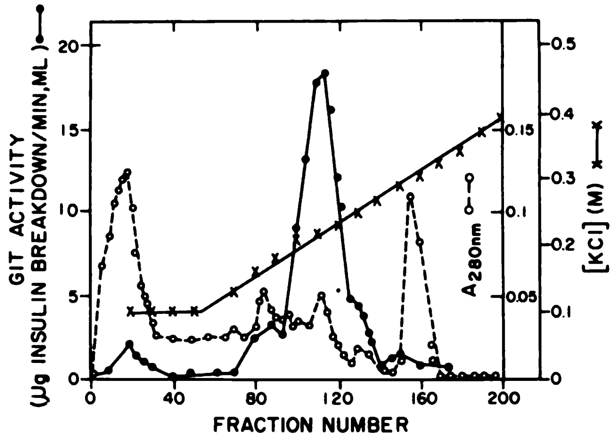
min. The pellet (microsomal fraction)
esuspended and centrifuged again un-
ie same conditions for 60 min. The
somes were then resuspended in an
iate volume of 0.44 *M* sucrose and
at −70° for subsequent assay. All
were at 0–4°.

purification of GIT was carried out
ding to the method of Ansorge *et al.*
ith the modification of the preparation
microsomal fraction described above.

ssay of GIT activity was modified from
f Ansorge *et al.* (2) and Chandler and
idani (8). When the microsomal frac-
vas used as the source of enzyme, it
homogenized with a Polytron (Brink-
Corp.) for 45 sec at 4.5 on the rheostat
assaying the enzyme activity. The
on mixture unless stated otherwise in
sults section consisted of: 15 μ*M* insu-
μl of ^{125}I-labeled insulin (5 μCi/5 ml)
England Nuclear); 4 μmoles of gluta-
(reduced form); 5 μmoles of EDTA;
00 μmoles of potassium phosphate
, pH 7.0, with a sufficient concentra-
f enzyme for a total volume of 1 ml.
nixture (without enzyme) was incu-
at 37° for 8 min followed by incuba-
with enzyme) at the same temperature
min with shaking. The reaction was stopped by cooling on ice and the addition of 1 ml of bovine serum albumin (4 mg/ml) and 2 ml of 10% TCA. The mixture was centrifuged at 2500 rpm for 15 min. One milliliter of the supernatant was taken from each tube, including the controls of total radioactivity and the blank, for liquid scintillation counting. One unit of enzyme activity was taken as that amount catalyzing the breakdown of 1 μg of insulin per minute at 37°. Protein was determined by the method of Lowry *et al.* (9), using bovine serum albumin as standard.

Results and discussion. As indicated in Methods, glutathione-insulin transhydrogenase was purified by a slight modification of the method described by Ansorge and his associates (2). Fourteen milliliters of the concentrated active fraction (80.5 mg of protein) obtained from the Sephadex G-75 column was placed on a 3.0 × 30-cm column of DEAE-Sephadex A-50 which had equilibrated with 0.1 *N* Tris buffer, pH 7.82, containing 2 m*M* 2-mercaptoethanol, and 0.1 *N* KCl. Elution was initiated with 100 ml of the same buffer and continued with a linear gradient of KCl (0.1–0.4 *M*) in 0.1 *M* Tris buffer, pH 7.82, containing 2 m*M* 2-mercaptoethanol using an LKB gradient former. The flow rate was 19 ml/hr,



1. Chromatography of glutathione-insulin transhydrogenase (GIT) fractions from Sephadex G-75 of
·Sephadex A-50. Fourteen milliliters of the concentrated active fraction (80.5 mg of protein) obtained from
tography on Sephadex G-75 was applied to a column (3.0 × 30 cm) of DEAE-Sephadex A-50 equilibrated
l *M* Tris buffer, pH 7.82, containing 2 m*M* 2-mercaptoethanol and 0.1 *M* KCl. The elution was begun with
of the same buffer and continued with a linear gradient of KCl (0.1–0.4 *M*) in 0.1 *M* Tris buffer, pH 7.82,
ercaptoethanol using an LKB gradient former. The flow rate was 16 ml/hr, and 5-ml fractions were
d at 4°.

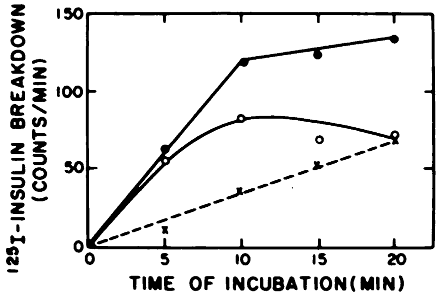


FIG. 2. Degradation of ^{125}I-labeled insulin as a function of time. One-hundred microliters of solution (44 µg of insulin and 100 µl of ^{125}I insulin (5 µCi/5ml) were mixed with 40 µl of 25 mM glutathione and 500 µl of 0.2 M potassium phosphate buffer, pH 7.0, containing 10 mM EDTA. The mixture was preincubated for 8 min at 37°. After this time (designated as 0 time in the figure) 0.632 µg of protein as GIT was added and the solutions incubated at 37°. Phosphate buffer was added in place of GIT in the blanks. See Methods for further details. (●), Total degraded insulin; (×), nonenzymatic degraded insulin; (O), GIT-catalyzed insulin breakdown (total amount of enzymatically degraded insulin minus that nonenzymatically degraded).

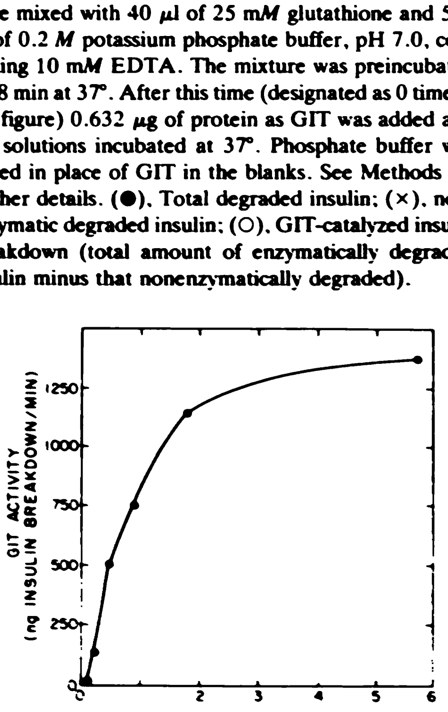


FIG. 3. Relationship of purified GIT amount (protein) and the rate of insulin degradation. See Methods for details of the assay.

with 5-ml fractions being collected at 4°. As can be seen from Fig. 1 a peak of enzyme activity was obtained between the 90th and 120th fraction. This enzyme was used to determine the relationships seen in Figs. 2 and 3.

Figure 2 shows the relationship of enzymatic to nonenzymatic degradation of ^{125}I-labeled insulin as a function of time. The greatest difference between the levels of enzymatic and nonenzymatic insulin degradation occurred at 10 min; thus, this time was selected for the incubation time for the standard assay. Use of shorter times (5 min) for assay did not alter the K_m values seen in the tables. A plot of the rate of enzymatic insulin breakdown versus the amount of GIT protein is linear only at low concentrations of enzyme (Fig. 3). In order to approximate zero-order reaction conditions and thereby have a quantitative assay for the amount of GIT present, different concentrations of insulin and glutathione were tested (Fig. 4). Concentrations of 4 mM glutathione and 15 µM insulin gave linear results up to 56 µg of microsomal protein. Polytron treatment of the microsomal fraction after homogenization with the Potter–Elvehjem homogenizer resulted in an increase in GIT activity from 2.7 to 8.0 units/mg of protein. Thus, the specific activity for the enzyme was measured after Polytron treatment in all subsequent assays in a reaction mixture containing 4 mM glutathione and 15 µM insulin and at concentrations of less than 56 µg of microsomal protein.

The specific activity of GIT was determined as a function of rat age (Fig. 5). The newborn rats were separated from their mothers immediately before sacrifice, with livers from the same littermates being pooled for each time point until the 12th day after birth subsequent to which the liv-

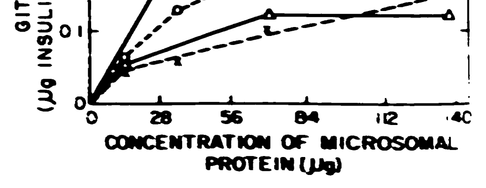


FIG. 4. Relationship of the concentration of microsomal proteins with GIT activity at different substrate concentrations. (×), 4 mM glutathione, 44 µg of insulin; (●), 4 mM glutathione, 88 µg of insulin; (○), 2 mM glutathione, 44 µg of insulin; (△), 2 mM glutathione, 88 µg of insulin. See Methods for further experimental details.

ers were assayed individually. Fetal rats had levels of GIT close to the adult (62-day) levels. However, upon birth the GIT specific activity was double that of the adult, with a gradual decay to adult levels by the second week after birth. It is of interest that the GIT activity in the Morris 7800 hepatoma was 3.1 ± 0.9 on the same basis as seen in Fig. 5. Thus, unlike many other enzymes which are low in activity in fetal liver and also in hepatomas (10), GIT activity in the fetal liver is the same as that in the adult during the latter stages of fetal life in contrast to the activity of the enzyme in the hepatoma which is less than half that seen in the adult. GIT activity in the host liver of the tumor-bearing animal was 5.5 ± 1.1 (average ± SEM of animals).

The effect of dietary and hormonal alterations on the specific activity of microsomal glutathione-insulin transhydrogenase can be seen in Table I. Increasing the protein content of the diet from 0 to 90% and allowing animals to eat such diets for a period of 10 days did not result in any change in the specific activity of GIT in microsomal protein. However, there was undoubtedly a total increase in the enzyme since other investigations (11) have demonstrated significant increases in the amount of microsomal protein per liver cell as the protein content of the diet increases. In animals fed a laboratory chow diet the administration of glucagon at the dose seen in Table I resulted in no significant change or a slight increase in the specific activity of the enzyme during the time interval investigated. Cortisone administration to adrenalectomized rats also had no effect on the specific activity of GIT in the microsomes 4 hr after its administration. However, as can be seen from the data in

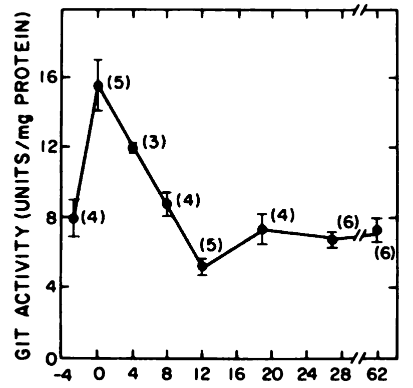


FIG. 5. The specific activity of GIT as a function of age in rats. The treatment of animals and the assay of GIT are described in the methods section. The numbers in parentheses indicate the number of animals utilized for each assay point beyond the 12th day. The numbers at the 12th and earlier days refer to the number of batches of pooled livers from littermates.

TABLE I. THE EFFECTS OF CORTISONE, GLUCAGON, AND DIFFERENT PROTEIN DIETS ON THE SPECIFIC ACTIVITY OF MICROSOMAL GLUTATHIONE-INSULIN TRANSHYDROGENASE.

Conditions	Units/mg of GIT microsomal protein[a]
0% Protein diet, 10 days	9.2 ± 2.9 (6)
12.5% Protein diet, 10 days	7.2 ± 1.9 (6)
60.0% Protein diet, 10 days	7.1 ± 1.0 (6)
90.0% Protein diet, 10 days	9.3 ± 2.3 (6)
Glucagon (1 mg/100 g B.W.), 4 hr	13.7 ± 0.8 (3)
Control, 4 hr	10.7 ± 1.6 (4)
Cortisone (10 mg/100 g body weight), adrenalectomized rats, 4 hr	8.4 ± 2.6 (4)
Control, adrenalectomized rats, 4 hr	8.0 ± 0.9 (4)

[a] Values represent the means ± standard deviation, with the number of rats used in parentheses. See text for further details.

TABLE II. THE EFFECTS OF CORTISONE AND GLUCAGON ON THE K_m AND V_{max} OF GLUTATHIONE-INSULIN TRANSHYDROGENASE.

Conditions	V_{max} (nmoles/min)[a]	K_m (μM)[a]	Microsomal protein (mg)
Glucagon (1 mg/100 g body weight), 4 hr	0.172 ± 0.038	11.2 ± 0.3	0.23
Control, 4 hr	0.174 ± 0.016	7.5 ± 2.0	0.18
Cortisone (10 mg/100 ǵ body weight), adrenalectomized rats, 4 hr	0.288 ± 0.078	12.5 ± 4.7	0.23
Control, adrenalectomized rats, 4 hr	0.686 ± 0.038	40.2 ± 14.5	0.21

[a] Values represent the means ± standard deviation of three rats. These kinetic assays were run with the same rats used in Table I. See text for further details. V_{max} values were the rates under conditions of zero-order kinetics (Fig. 4).

Table II, while glucagon did not significantly change the K_m of GIT after administration, that of the enzyme in the adrenalectomized animal exhibited significant differences from the control. Hepatic microsomal GIT of adrenalectomized animals showed a fourfold increase in its K_m and a two- to threefold increase in V_{max} as compared to that of the enzyme in control or glucagon-treated intact animals. Administration of cortisone to adrenalectomized animals resulted in a drop of both the K_m and the V_{max} of hepatic microsomal GIT to essentially normal levels within 4 hr.

While it is relatively easy to understand the insensitivity of the levels of GIT to dietary and hormonal changes, the alteration in V_{max} and K_m in the adrenalectomized animal presents some interesting possibilities. Previous theoretical (12) and kinetic (13, 14) studies of enzymes associated with membranes have demonstrated alterations in enzymatic behavior resulting from the membrane environment of the enzyme. It is interesting to speculate that in the adrenalectomized animal, the change in hormonal environment may result in changes in membrane synthesis and structure leading to the altered kinetic constants of this microsomal enzyme. The effect of hormones on membrane synthesis and membrane enzymes has been previously reported both for the liver and the kidney (15). The effect of other environmental changes on the kinetic constants of GIT is presently under investigation in this laboratory.

Summary. The dietary and hormonal regulation of the level of glutathione-insulin transhydrogenase in rat liver was investigated in these studies. In order to make valid comparisons, the assay of glutathione-insulin transhydrogenase was performed at near zero-order kinetics wherein enzyme rate was proportional to enzyme amount. Changing the protein content of the diet or administration of glucagon or cortisone did not significantly affect the specific activity of glutathione-insulin transhydrogenase in microsomes from rat liver. However, the V_{max} and K_m of this enzyme in the livers of adrenalectomized rats were increased three- and fourfold over these values in microsomes from normal liver. Administration of cortisone resulted in a return to the normal kinetic constants of microsomal GIT within 4 hr.

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s Outflow of the Hormones Secreted by the Rat Pituitary Autotransplanted Beneath the Kidney Capsule[1] (39452)

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at hypophysis, when transplanted the kidney capsule, will secrete ele- ounts of prolactin for an extended 1–3). A low level of luteinizing hor- H) and follicle stimulating hormone elease, which can be enhanced by treatment with LH-releasing hor- ppears to persist for at least 16 days renal transplantation (4). The vas- nections of the transplant with the circulation have not been ade- defined, although the renal artery implicated as the efferent vessel prospect of using this ectopic pitui- paration as a model to investigate se of LH and prolactin in response us stimuli led us to determine its hormones were secreted into the in. In these studies, jugular venous the hormones were taken to repre- ir concentration in the peripheral n.

als and methods. Adult Holtzman -Dawley) male or female rats were sectomized under ether anesthesia rapharyngeal approach and studied such or after pituitary autotrans- n. In the latter case, the whole pi- recovered from a trap bottle in the ine, was placed under the left kid- ule immediately after hypophysec- small number of intact regularly male rats in diestrus was also stud- rats were kept under controlled –10 hr light:dark) and temperature onditions, with free access to Purina ry chow and tap water. At various after the operation the rats were lightly anesthetized with ether, and 1 ml or less of blood was removed by jugular venipuncture. In addition, one group of pituitary autotransplanted rats was killed 8 days after operation, just after sampling the jugular vein, by exsanguination through the left renal vein. The remaining autotransplanted rats were killed 4 weeks after operation, when blood samples were taken simultaneously from the jugular, right, and left renal veins.

Completeness of hypophysectomy was ascertained by macroscopic examination of the pituitary fossa and atrophy of the adrenal glands and gonads. The blood samples were permitted to clot at room temperature, then centrifuged at 4°, and the serum was stored at $-20°$ until assayed. The serum from each sample was assayed in duplicate for prolactin and LH by specific radioimmunoassays (6, 7). The LH values were expressed in terms of rat LH standard LER-1240-2 (0.6 × NIH-LH-S1); prolactin values were expressed in terms of rat prolactin standard LER-1382-82 (45.7 IU/mg).

Results. The results are summarized in Table I. The LH levels in the jugular vein serum of the hypophysectomized-pituitary autotransplanted rats were not significantly different from those found in the renal vein serum and did not seem to be affected by the sex of the host or by the interval after operation. Although the values were about half of those found in the intact diestrous females, none of them fell below the minimal detectable level of the assay (0.15 ng/ml). In the hypophysectomized rats, however, all the values were found to be below the minimal detectable level by the third week after operation, although a mean value of 0.22 ng/ml was found 1 week after operation.

The prolactin levels in the jugular vein serum of the hypophysectomized-pituitary autotransplanted rats were also indistin-

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rved.

TABLE I. Levels of Prolactin and LH in the Jugular and Renal Vein Serum of Rats after Hypophysectomy or Hypophysectomy Plus Pituitary Autotransplantation.[a]

Experimental condition	Number of rats[b]	Interval after operation (days)	Serum LH levels (ng/ml)		Serum prolactin levels (ng/ml)	
			Jugular	Renal[c]	Jugular	Renal[c]
Intact, diestrus	4 (F)	–	0.61 ± 0.11	–	7.0 ± 2.0	–
Hypophysectomy	4 (F)	7	0.22 ± 0.01	–	–	–
		21	<0.15[d]	–	–	–
	5 (F)	7	–	–	1.51 ± 0.13	–
		14	–	–	1.55 ± 0.05	–
		21	–	–	1.49 ± 0.03	–
		28	–	–	1.52 ± 0.05	–
Hypophysectomy-pituitary autotransplantation	5 (M)	8	0.30 ± 0.3	0.29 ± 0.03	76.1 ± 29.6	30.2 ± 6.2
	5 (F)	8	0.32 ± 0.04	0.29 ± 0.04	51.5 ± 11.0	48.9 ± 6.6
	10 (F)	0[e]	2.85 ± 0.25	–	53.0 ± 8.7	–
		7	0.32 ± 0.03	–	73.3 ± 5.1	–
		14	0.38 ± 0.04	–	45.4 ± 3.4	–
		21	0.36 ± 0.04	–	44.1 ± 2.4	–
		28	0.36 ± 0.03	0.39 ± 0.05[f]	41.7 ± 3.8	48.3 ± 5.5[g]

[a] Values for prolactin and LH are in all cases means and SEM.

[b] Sex of the subjects indicated in parentheses.

[c] The vein draining the pituitary transplant-bearing kidney; in all cases this was the left one. The *LH standard* was LER-1240-2 (0.6 × NIH-LH-S1); the *prolactin* standard was LER-1382-82 (45.7 IU/mg).

[d] The minimal detectable level was 0.15 ng/ml; in all cases the level was below this minimum.

[e] Sampled immediately before operation.

[f] The LH level in the right renal vein was 0.4 ± 0.04.

[g] The prolactin level in the right renal vein was 42.7 ± 3.7.

guishable from those found in the renal veins of either kidney and were also unaffected by the sex of the donor or the interval after operation. They were many-fold higher than the values found in either the intact diestrous rats or those subjected only to hypophysectomy.

Discussion. The hormones secreted by the transplanted pituitary, thus, do not appear to be secreted into the renal vein. The transplant is apparently vascularized by the blood vessels of the capsule, to which it forms a firm attachment. The blood supply of the renal capsule has not been exactly identified in the rat, but it is probable that the major venous drainage is the same as that of the parietal peritoneum, i.e., the phrenico-abdominal and cranial abdominal veins on the right and left sides, respectively. These empty into the inferior vena cava just cranial to the renal veins (8).

The finding of high serum prolactin levels in hypophysectomized-pituitary autotransplanted rats in neither surprising nor new. The fact that the LH levels were slightly but significantly higher than after simple hypophysectomy, up to at least 4 weeks after operation, however, suggests that LH secretion is not completely prevented by separation of the pituitary from its normal connections to the hypothalamus. This conclusion is also supported by the recent finding of low basal levels of LH in the hypophysectomized rat bearing a homotransplanted pituitary from a long-term ovariectomized donor (9); these levels, however, were not compared with those of rats subjected to hypophysectomy alone. The most immediate importance of the finding that LH can be secreted in small amounts by the transplanted pituitary is the reservation which this imposes on the experimental use of the transplanted pituitary to study the effects of prolactin separate from any possible effect of LH.

Summary. In adult female or male rats subjected to hypophysectomy and autotransplantation of the pituitary beneath the renal capsule, the levels of prolactin in the renal vein of the pituitary-bearing kidney were not significantly different from those found either in the renal vein of the contralateral kidney or in the jugular vein. LH levels were slightly but highly significantly higher than those found in hypophysectomized rats; the levels found in the two renal veins and the jugular vein were also not significantly different from one another.

We express out thanks to Helen Wilk and Rosa Garnett for their assistance with the animals and the preparation of this paper, respectively. The hormones and antibodies used for the assay of LH and prolactin were generously donated by Drs. G. D. Niswender, J. D. Neill, and L. E. Reichert and by the Program Officer, NIH Hormone Distribution Program.

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Developmental Patterns of Acid Hydrolases during Differentiation of Fetal Mouse Skin (39453)

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Acid phosphatase and β-glucuronidase are considered as a group of lysosomal enzymes in other tissues (1, 2). However, these enzymes demonstrate opposite distributions in the vertical strata of the epidermis. Acid phosphatase activity is greater in the upper layers of the epidermis (3, 4), while β-glucuronidase is concentrated in the lower layers (5). The differential distribution of acid phosphatase and β-glucuronidase in the epidermal strate may be correlated with progressive differentiation of keratinocytes. The inverse relationship in the development of these two hydrolytic enzymes is reflected in an increasing ratio of acid phosphatase to β-glucuronidase activity during epidermal regeneration and maturation during wound healing. The migrating epithelial tip observed during wound healing contains a relative paucity of acid phosphatase (4) but an abundance of β-glucuronidase (5). The ratio of acid phosphatase to β-glucuronidase activity appears to represent an indicator for the degree of epidermal differentiation.

The present studies were undertaken to determine whether progressive differentiation of fetal epidermis was accompanied by changing patterns of acid phosphatase and β-glucuronidase activity.

Materials and Methods. Inbred strain A/Jax mice (Jackson Laboratory, Bar Harbor, Maine) were bred, and the presence of the vaginal copulation plug was taken as Day 0 of pregnancy. Pregnant mice were sacrificed at Days 14, 15, 17, and 19 of gestation, and the fetuses were removed quickly. The fetuses and newborn mice (0–14 days) were decapitated, and the heads were frozen in liquid nitrogen. The heads of the fetuses and neonate mice were sectioned in a coronal plane at 24-μm thickness and lyophilized at −20° in a cryostat. The freeze-dried tissues were stored in a vacuum tube at −20° until the enzymes were assayed. Epidermal segments from the scalp skin were obtained from the freeze-dried sections by microdissection, weighed (0.3–0.5 μg) on a quartz fiber microbalance, and transferred into microtest tubes (2.5 × 25 mm) for subsequent enzyme analyses. Stratum cornium was excluded from the epidermal samples.

Acid phosphatase activity was assayed by the fluorometric method with α-naphthyl phosphate as the substrate (6). Optimal assay conditions for acid phosphatase activity were previously determined for the skin (3). The reaction mixture consisted of 20 m*M* α-naphthyl phosphate, 0.05 *M* acetate buffer (pH 5.3), 2 m*M* $MgCl_2$, and 0.02% bovine serum albumin. Seven microliters of reagent mixture was added to 0.3–0.5 μg of fetal or neonatal epidermis in microtest tubes and incubated for 60 min at 37°. The standard tubes contained 1–2 nmoles of α-naphthol per tube, and the blank tubes contained reagent mixture only. After incubation, a 5-μl aliquot was diluted to 1 ml with 0.5 *N* NaOH, and the fluorescence of the α-naphthol formed by the enzyme reaction was measured in a fluorometer equipped with the glass filter system. The primary filter was Corning Glass No. 5860 and the secondary filter consisted of No. 3387 and 4308.

β-Glucuronidase activity was measured by the fluorometric method with 4-methylumbelliferone β-D-glucuronide as substrate (7). Optimal assay conditions were determined previously with epidermal tissue (5) and found to be: 0.1 *M* acetate buffer (pH 3.5), 1.5 m*M* 4-methylumbelliferone β-D-glucuronide, and 0.02% bovine serum albumin. Approximately 0.5 μg of microdissected epidermal segments were incubated in 7 μl of reagent mixture for 60 min at 37°.

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TABLE I. HYDROLYTIC ENZYME ACTIVITIES IN DEVELOPING MOUSE EPIDERMIS.

	Acid phosphatase[a]			β-Glucuronidase[b]			Activity ratio[c]
;e	Mean ± S.D.	Range		Mean ± S.D.	Range		
;e							
14				15.0		(1)	
15	0.15 ± 0.05	0.08-0.26	(8)[d]	13.0 ± 1.4	10.5-15.3	(6)	12
17	0.37 ± 0.12	0.22-0.57	(7)	6.5 ± 1.8	4.6-9.5	(6)	57
19	0.87 ± 0.08	0.75-0.96	(6)	6.5 ± 0.9	5.1-7.2	(6)	133
al age							
0	1.28 ± 0.24	0.80-1.22	(14)	8.4 ± 2.6	5.0-13.6	(9)	152
2	1.48 ± 0.33	1.13-1.89	(4)	7.6 ± 0.9	6.4-8.7	(4)	194
4	0.81 ± 0.13	0.76-0.97	(4)	8.1 ± 1.6	6.2-10.5	(4)	100
7	0.77 ± 0.14	0.63-1.01	(8)	8.1 ± 2.4	4.3-12.2	(8)	95
14	0.50 ± 0.07	0.46-0.62	(4)	7.2 ± 0.5	6.4-7.6	(4)	69

tivity is expressed as moles per hour per kilogram dry weight.

tivity is expressed as millimoles per hour per kilogram dry weight.

notes the ratio of acid phosphatase activity to β-glucuronidase activity.

mber of animals used (each assayed in quintuplicate); number of litters used were two to nine each.

tandard tubes containing 5-20 pmoles
methylumbelliferone and the blank
containing reagent mixture only were
d through the procedure. The fluores-
of the product of enzyme reaction was
ıred in a fluorometer equipped with
ng Glass No. 5860 as the primary fil-
ıd No. 3387 and 5543 as the secondary

ults. Acid phosphatase and β-glucu-
ase activities, as a function of develop-
ıl age, in the epidermis are summa-
in Table I. The level of acid phospha-
ıctivity was low in the epidermis on
ı5 of gestation, the activity level being
ı0% of the maximum activity found in
arly neonates. On the other hand, β-
ronidase exhibited its highest activity
ıys 14 and 15 of gestation. Acid phos-
se activity increased gradually from in-
:rine Day 15 into neonatalhood. β-
ıronidase activity fell to half of the
ı4 level by Day 17 of gestation and
ained the low level during the closing
of fetal development and the neonatal
d. The results demonstrated an oppo-
levelopmental pattern of the two hy-
ses during epidermal development.

: ratio of acid phosphatase to β-glucu-
ase activity (A/G ratio) was low on
ı5 of gestation and increased markedly
after, achieving a maximum on Day 2
artum (Table I). The A/G ratio was 12
ay 15 of gestation and approximately 200 on Day 2 postpartum. The A/G ratios decreased during the second week after birth.

TABLE II. HYDROLYTIC ENZYME ACTIVITIES IN VERTICAL STRATA OF NORMAL ADULT GUINEA PIG EPIDERMIS[a].

Layer	Acid phosphatase[b]	β-Glucuronidase[c]	Activity ratio[d]
Keratin	0.76	12.1	63
Upper half	1.35	13.5	100
Lower half	0.43	36.1	12

[a] From Ref. (4) and (5).

[b] Activity is expressed as moles per hour per kilogram dry weight.

[c] Activity is expressed as millimoles per hour per kilogram dry weight.

[d] Denotes the ratio of acid phosphatase activity to β-glucuronidase activity.

Discussion. Microanalytical studies have revealed preferential localization of acid phosphatase in the upper layers of the epidermis (4) and β-glucuronidase in the lower layers (5) (Table II). The unique pattern of acid phosphatase concentration in the granular layers of epidermis indicates indirectly that it is involved in the process of keratinization and cellular autolysis (8). The high level of β-glucuronidase activity in the lower layer suggests that this enzyme may be related to cellular proliferation and that the basal cells may be sites for more active turnover of glycosaminoglycan. Therefore, changes in the relative ratio of acid phosphatase to β-glucuronidase activity can be correlated with progressive differentiation in whole epidermis.

Acid phosphatase activity on Day 15 of gestation is only 10% of that on Day 2 postpartum. β-Glucuronidase exhibits a high activity level on Days 14 and 15 of gestation. The activity ratio of acid phosphatase to β-glucuronidase (A/G ratio) increased 11 times from Day 15 to Day 19 of gestation, and a high A/G ratio persisted between intrauterine Day 19 and neonatal Day 2. These data may reflect a rapid expansion of epidermal differentiation during the late days of fetal development and early neonatal life.

Summary. The activities of acid phosphatase and β-glucuronidase were assayed in fetal and neonatal mouse epidermis by microanalytical methods. The level of acid phosphatase activity was low in the epidermis on Day 15 of gestation. Acid phosphatase activity increased 10-fold between Day 15 of gestation and neonatalhood. On the other hand, β-glucuronidase activity was high on Days 14 and 15 of gestation and low after Day 17 of gestation. The relative ratio of acid phosphatase to β-glucuronidase activity appeared to represent a marker for the degree of differentiation in whole epidermis.

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in Vitro Generation of a Chemically Reactive Metabolite of 2,5,2′,5′-Tetrachlorobiphenyl by Rhesus Monkey Liver Microsomes (39454)

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family of compounds known as polyiated biphenyls (PCBs) has been used istry since the 1930's as heat exchangransformers, plasticizers in paints and ves, electrical and hydraulic fluids, retardant coatings for woods, and vappressants for insecticides, to name a 'he properties that make PCBs suita- these uses, such as high temperature nce to decomposition, also make :xtremely stable compounds when acılly freed into the environment. Re-, several reports have shown that are widespread global contaminants CBs consist of a large mixture of isoınd it has only been recently that pure 's were available for metabolic studıe such isomer, 2,5,2′,5′-tetrachloroıyl (TCB), has been shown by Van *et al.* (2) to be rapidly metabolized in to 3-hydroxy-2,5,2′,5′-tetrachlorobi-. In other species, such as rabbits, er *et al.* (3) have demonstrated that ,5′-tetrachlorobiphenyl is metabohrough an arene oxide pathway to olic products consisting of trans-dihyls and monohydroxy metabolites. Re-, Hsu *et al.* (4) have shown the pres-of trans-dihydrodiols, monohydroxy, oxy and transdihydrotriols in urine of and adult rhesus monkeys given an dose of the pure isomer. Dihydrodiols hydrotriols are thought to be derived vely from an arene oxide intermediany members of this class of intermehave been shown to be alkylating with carcinogenic potential (5). In species the liver microsomal fraction en shown to be the principle site of tion of arene oxides. The presently ed study demonstrates that a metabo-2,5,2′,5′-TCB capable of covalently g to macromolecules is formed *in vi*-monkey liver microsomes.

Materials and methods. Tritiated 2,5,-2′,5′-TCB ([^{3}H]TCB) was prepared by the method of Hutzinger and Safe (6), and liver microsomes were obtained from an adult female rhesus monkey (7). The basic incubation mixture consisted of 5 μmoles of NADP, 3 μmoles of ATP, 10 μmoles of glucose-6-phosphate, 25 μmoles of $MgCl_2$, and 2.5 IU of glucose-6-phosphate dehydrogenase in phosphate buffer (pH 7.4). To this NADPH generating system was added 0.3 ml of a 33% microsomal suspension (0.3 ml of microsomes from 0.1 g of liver) either active or as heat-deactivated (100° for 10 min) controls. The microsome-cofactor system was divided into four groups, each of which had heat-inactivated microsomes serving as a control. In Group 1 water was added; in Group 2 glutathione (GSH) (1 μmole) was added; Group 3 received 0.2 ml of postmicrosomal supernate (0.3 ml from 0.1 g of homogenized liver); and Group 4 received both 0.2 ml of postmicrosomal supernate and 1 or 10 μmoles of GSH. Substrate was added as [^{3}H]TCB (1.25 μCi, 12 μCi/mg) in 10 μl of methanol. The final volume was 3.7 ml. The flasks were stoppered and incubated at 37° for 1.0 hr in a shaking water bath (120 strokes/min).

The incubation was stopped by the addition of 1.25 ml of ice-cold 20% trichloroacetic acid (TCA). The resulting 5% TCA solution was held at 4° for 1 hr. The precipitate (Fig. 1) was collected by centrifugation (800*g*) and washed three times with ice-cold 5% TCA. TCA washes of each group were combined (Fraction I) for later testing for the presence of TCA soluble metabolite(s). The resulting protein-RNA pellet was washed (8×) with methanol (60°) until all extractable radioactivity was removed and the last wash had only background levels. The methanol extracts were combined and saved for later metabolite determination

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served.

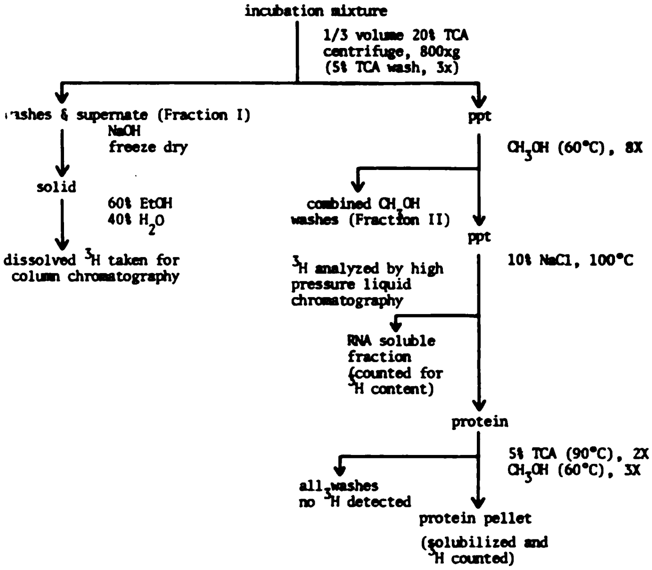
incubation mixture
1/3 volume 20% TCA
centrifuge, 800xg
(5% TCA wash, 3x)
washes & supernate (Fraction I)
NaOH
freeze dry
solid
60% EtOH
40% H2O
dissolved 3H taken for column chromatography
ppt
CH3OH (60°C), 8X
combined CH3OH washes (Fraction II)
3H analyzed by high pressure liquid chromatography
ppt
10% NaCl, 100°C
RNA soluble fraction (counted for 3H content)
protein
5% TCA (90°C), 2X
CH3OH (60°C), 3X
all washes
no 3H detected
protein pellet
(solubilized and 3H counted)

FIG. 1. Flow chart for the extraction of liver microsomes incubated with [^{3}H]2,5,2′,5′-tetrachlorobiphenyl.

(Fraction II). The pellet was dried with ether and extracted with 10% NaCl (1 hr, 100°) followed by two extractions with 10% NaCl ($^1/_2$ hr, 100°) to dissolve RNA (8, 9). The sodium chloride extracts were combined and sampled for ^{3}H content. The protein pellet was further extracted (2×) with 5% TCA (90°) to extract any remaining RNA and wahsed three times with methanol (60°) to eliminate any trapped [^{3}H]TCB that might have been released with the RNA extraction. The resulting protein pellet was dissolved in Unisol and the radioactivity counted after the addition of Unisol-Complement. The NaCl, TCA, and methanol washes were sampled or added whole to 10 ml of Aquasol and counted on a Packard Tri-Carb 3375 liquid scintillation spectrometer with an automatic external standard. This complete procedure when conducted with 5 mg of bovine serum albumin substituted for microsomes showed no statistical difference between normal and heat inactivated protein in the adsorption of [^{3}H]TCB.

Chromatography of the initial methanol extract (Fraction II) of the TCA insoluble pellets was carried out on a Waters Associates 6000 psi high-pressure liquid chromatograph (C_{18}-corasil, 80% CH_3OH-20% H_2O) equipped with two model 6000A pumps, solvent programmer, and ultraviolet and refractive index detector. The initial TCA washes (Fraction I) of the protein-RNA pellet were neutralized with NaOH and freeze-dried. The resulting solid was extracted serially with hexane, ether, methanol, and finally ethanol:water (3:2). The ethanol:water soluble material was chromatographed on silica gel 60 (70–230 mesh) (E. Merck) using ethanol:water (3:2) as the solvent at a flow rate of 1.0 ml/min.

Results. Distribution of the [^{3}H]TCB in homogenate fractions. The exhaustive methanol extraction (Fraction II) removed greater than 98% of the [^{3}H]TCB from the initial TCA precipitates of the normal microsomes and the heat inactivated controls in all groups. Approximately 0.2% of the [^{3}H]TCB remained bound to macromolecules in Group 3 with 0.05% represented as adsorbed material (control homogenates). Introduction of GSH into an active generating system (Groups 2 and 4) increased the TCA soluble [^{3}H]TCB by 1.0 to 0.5% above a standard level of approximately 0.4% in all other groups (Table I). The low conversion rate is due to the stability of the TCB molecule. *In vivo* conversion to excretable metabolites takes place at only approximately 1%/day (10).

In vitro generation of PCB metabolite capable of binding to macromolecules. As can een in Table II, homogenates of heat ivated microsomes bound some radio- ity that was not extracted. This material onsidered to be adsorbed to the macro- cules and was used as an adsorption rol. The water incubation (Group 1) in h only the microsomes, substrate, and PH generating system were present, luced a significant quantity of tritium d to the protein fraction compared to heat inactivated controls. When GSH up 2) was added to this system, no ficant change in bound tritium could be cted. When microsomal supernate up 3) was added, a significant increase e amount of tritium bound to protein noted. The addition of either 1 μmole 0 μmoles of GSH to the incubation medium containing microsomal supernate (Group 4) eliminated the significant increase in tritium bound to proteins. The incubations with microsomal supernate strongly increased binding of tritium to the RNA fraction. A reduction of tritium bound to RNA was noted by the addition of either 1 or 10 μmole of GSH to the reaction flask (Table III). In both instances the reduced level was similar to that occurring in Group 2.

LE I. The Amount of [^{3}H]TCB (Metabo- ed and Unmetabolized) Appearing in the A Soluble Fraction (Fraction I) of Micro- ies Incubated with [^{3}H]TCB.

p	^{3}H as a percentage of substrate ±SD	
H_2O	0.52 ± 0.11	(7)[a]
H_2O boiled	0.31 ± 0.06	(5)
GSH (0.31 mg)	1.24 ± 0.23	(7)
GSH boiled	0.40 ± 0.12	(5)
MS[b]	0.43 ± 0.10	(7)
MS boiled	0.35 ± 0.08	(5)
MS + GSH (0.31 mg)	1.09 ± 0.19	(7)
MS + GSH boiled	0.37 ± 0.06	(5)

umber of incubations.
icrosomal supernate.

In vitro generation of polar PCB metabolites. The methanol extracts (Fraction II) of the 5% cold TCA precipitate were chromatographed on corasil-C_{18} column (1.0 ml/min, 100 psi) using 20% H_2O–80% CH_3OH as the solvent. This system has been used in our laboratory to separate unmetabolized TCB from its hydroxylated metabolites (4). Mono-, di-, and trihydroxylated metabolites all elute with the solvent front in this system while TCB elutes several fractions later. Essentially no metabolized TCB could be found in the methanol extract of the incubation with microsomal supernate (limit of detection about 0.5%).

The chromatography of the TCA soluble material from Group 2 (Fraction I) on silica gel using ethanol:water (60:40) as the solvent showed an elution pattern different from free [^{3}H]TCB indicating a possible metabolic product. The elution volume for free [^{3}H]TCB mixed with GSH and TCA was 45 ml while the [^{3}H]TCB extracted from the TCA soluble freeze-dried material had an elution volume of 58 ml on the same

LE II. [^{3}H]TCB Bound to Protein Resulting from Incubation of [^{3}H]TCB and Monkey Liver Microsomes in a NADPH Generating System.

Substrate added to homogenate		dpm Bound to protein ±SD	
Group 1	H_2O	2,255 ± 548[a]	(7)[i]
	H_2O (heated microsomes)	1,218 ± 246[a]	(5)
Group 2	GSH	2,872 ± 258[b,g]	(7)
	GSH (heated microsomes)	1,520 ± 77[b]	(5)
Group 3	MS[h]	4,782 ± 539[c,g]	(7)
	MS (heated microsomes)	1,260 ± 76[c]	(5)
Group 4	MS + GSH (.31 mg/flask)	2,061 ± 486[d,f]	(7)
	MS + GSH (3.1 mg/flask)	1,934 ± 238[e]	(7)
	MS + GSH (.31 mg/flask) (heated microsomes)	1,084 ± 138[d,e]	(5)

te: [a] $p \leq .005$; [b] $p \leq .0001$; [c] $p \leq .0001$; [d] $p \leq .001$; [e] $p \leq .0001$; [f] $p \leq .0001$; [g] $p \leq .0001$ (Student's *t* test to determine *p* value); [h] microsomal supernate; [i] number of incubations.

TABLE III. ^{3}H-TCB BOUND TO MICROSOMAL RNA AFTER INCUBATION OF ^{3}H-TCB WITH RHESUS MONKEY LIVER MICROSOMES IN A NADPH GENERATING SYSTEM.

Substrate added to homogenate		dpm Bound to microsomal RNA ±SD	
Group 1	H_2O	302 ± 47^a	$(7)^i$
	H_2O (heated microsomes)	107 ± 13^a	(5)
Group 2	GSH	$201 \pm 71^{b,g}$	(7)
	GSH (heated microsomes)	68 ± 34^b	(5)
Group 3	MS^h	$468 \pm 88^{c,f,g}$	(7)
	MS (heated microsomes)	41 ± 17^c	(5)
Group 4	MS + GSH (.31 mg/flask)	$154 \pm 79^{d,f}$	(7)
	MS + GSH (3.1 mg/flask)	195 ± 93^e	(7)
	MS + GSH (.31 mg/flask) (heated microsomes)	$57 \pm 13^{d,e}$	(5)

Note: ^{a} $p \leq .0001$; ^{b} $p \leq .005$; ^{c} $p \leq .0001$; ^{d} $p \leq .02$; ^{e} $p \leq .01$; ^{f} $p \leq .0001$; ^{g} $p \leq .0001$; Student's *t* test used to determine *p* value; ^{h} microsomal supernate; ^{i} Number of incubations.

column (Fig. 2). Exhaustive extraction of the freeze-dried TCA soluble material with hexane (60°) and ether (35°) failed to extract an appreciable quantity (10%) of tritium, indicating that the [^{3}H]TCB is not a mono-, di-, or triol, all of which are freely soluble in ether. The remaining tritium (90%) was solubilized by using methanol (60°) (but not methanol, 20°) or with ethanol:water (60:40). No such [^{3}H]TCB could be found in the freeze-dried TCA fraction of the heat-inactivated GSH incubation (Group 2).

Discussion. From the findings of Gardner *et al.* (3) and Hsu *et al.* (4), it has become increasingly apparent that the lower chlorinated PCBs containing adjacent hydrogens can readily be metabolized through the chemically active arene oxide intermediate. Jensen *et al.* (11) has shown that even higher chlorine isomers are metabolized to mono-oxygenated metabolites through a mechanism not yet fully characterized. Such metabolites can, and obviously do, form conjugated metabolites with other biological molecules. Proteins and RNA exposed to *in vitro* generation of a possible arene oxide intermediate of TCB have been shown here to covalently bind [^{3}H]TCB to a significant extent. Such reactions could conceivably lead to alterations in the macromolecular structure at the site of alkylation. The fact that hexane and ether were able to extract only 10% of the radioactivity from the TCA soluble material (Fraction I, Group 2) implies that the bulk of the radioactivity in the freeze-dried TCA soluble material (about 1% of the total substrate ^{3}H) was either more polar than the previously isolated triols or conjugated to very polar materials such as GSH. This would account for its initial solubility in the TCA soluble fraction. Comparatively, 0.2% of the total substrate was found to be covalently bound to macromolecules when GSH was not present (Group 3, Table II). This represents a 20% binding of metabolically converted products.

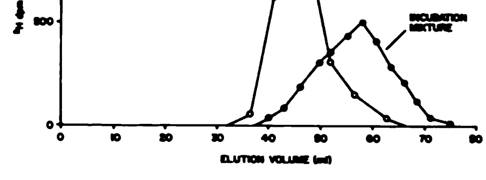

FIG. 2. Chromatography of [^{3}H]TCB, TCA, and GSH (○——○) and the TCA soluble material from the incubation (Fraction I, Group 2) (●——●) on a silica gel-60 column using 60% ethanol–40% water as the eluting solvent.

Kimbrough *et al.* (12) administered higher chlorine isomer mixtures of PCBs to 200 female rats for 2 years and observed a 14% incidence of tumors (vs 1% controls) and a 78% incidence of neoplastic nodules in the liver (vs 0% controls). It is conceivable that these tumors were due to the interaction of arene oxide-like intermediates with the genetic material at the cellular

Other investigators have also ob-
neoplastic liver nodules in rats (13)
ce (14). These arene oxides are usu-
tremely reactive as is evident by the
t tumors related to PCB administra-
re observed only in the liver where
ne oxide is presumably generated. In
periments, the active metabolite ap-
sufficiently nonreactive to leave the
synthesis and react with supernate
. This reaction was prevented by the
n of glutathione which presumably
ates the active metabolite before it
with protein. The fact that none of
at deactivated homogenates bound
uantities of radioactivity to proteins
H indicates that metabolism is re-
to achieve this binding capability.
ly materials heated were the micro-
suspensions and not the glucose-6-
ate or the microsomal supernate.
ore, the microsomal supernate alone
ot have the capacity to generate the
e intermediate, and the NADPH
ting system is completely intact in the
enate mixture.

fact that PCBs are so widespread in
ironment makes it extremely impor-
at we understand the mechanism by
these materials are toxic. When adult
monkeys were fed 2.5 ppm PCB in
t, skin lesions, abnormal menstrual
difficulty in maintaining pregnancy,
normal offspring were observed (15).
nceivable that certain segments of the
population are exposed to PCBs at
that may well exceed 2.5 ppm. The
tly reported study indicates that some
omers may be metabolized through a
e arene oxide intermediate which has
ential to bind to macromolecules and
eir character. These data suggest that
vill continue to be a significant health
for many years to come.

mary. Incubation of tritiated 2,5,-
trachlorobiphenyl with normal mon-
er microsomes in a NADPH-generat-
tem results in the formation of active
lite(s) of the [^{3}H]2,5,2′,5′-tetrachlorobiphenyl capable of covalently binding to RNA and protein isolated from the incubation mixture. The metabolite is not formed when the control microsomes are held at 100° for 10 min prior to incubation. The addition of microsomal supernate to the solution causes an increase in the binding of the active metabolite to macromolecules while the addition of glutathione to the incubation medium significantly inhibits this increase.

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Developmental Studies on Glucosamine Metabolism (39455)

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Glucosamine and its derivatives are unique and obligatory structural components of many biologically important macromolecules, including membrane glycoproteins and glycolipids, plasma glycoproteins, and glycosaminoglycans. During postnatal development, from birth to the early adult stage, a great deal of rapid growth and maturation occurs. One might expect that this would be reflected in an increased rate of synthesis of various hexosamine-containing macromolecules since these are components of all cells, tissues, and biological secretions. This increased synthetic rate should be reflected, in turn, in the rate of synthesis of glucosamine and its derivatives.

The present paper reports the results of assays of the activities of two key hepatic enzymes involved in hexosamine synthesis and conversion during postnatal development. In addition, the hepatic concentrations of two glucosamine metabolites involved in the feedback regulation of these enzymes were measured. Specifically, the enzymes examined were L-glutamine:D-fructose-6-phosphate aminotransferase (AT) and UDP-*N*-acetyl-glucosamine 2′-epimerase (EP). The metabolites measured were UDP-*N*-acetylglucosamine (UDP-GlcNAc) and CMP-*N*-acetylneuraminic acid (CMP-NANA).

Materials and methods. Animals. Male, Wistar-King A, rats bred, born, and raised in this laboratory were maintained on standard laboratory chow and housed until used in environmentally controlled animal quarters.

Chemicals. UDP-*N*-acetylglucosamine, *N*-acetylneuraminic acid, and fructose-6-phosphate were purchased from Sigma. All other chemicals were of the highest quality available from commercial sources.

Enzyme preparation. Rats were anesthetized with ether and the livers were exposed through an abdominal incision. Blood was withdrawn from the abdominal aorta for seromucoid determinations. The livers were perfused via the portal vein with an ice-cold solution of 0.1 *M* phosphate buffer, pH 7.5; then were removed, blotted, weighed, and homogenized in 2 vol of ice-cold 0.1 *M* phosphate buffer-1 m*M* EDTA, pH 7.5, for 1 min using a Polytron homogenizer Model 10ST operated at 10,000 rpm. The homogenate was centrifuged for 10 min at 20,000*g* and the postmitochondrial supernatant was removed and centrifuged for 60 min at 105,000*g*. The clear, middle portion of this supernatant served as the source of the enzyme activities.

Assay of L-glutamine-D-fructose-6-phosphate aminotransferase. L-glutamine:D-fructose-6-phosphate aminotransferase (AT) was assayed as previously described (1) according to the method of Kornfeld (2). Each reaction mixture contained 10 m*M* fructose-6-phosphate, 10 m*M* glutamine, 1 m*M* EDTA, 40 m*M* sodium phosphate buffer, pH 7.5, and 0.1 ml enzyme extract in a total volume of 0.5 ml. After incubation at 37° for 60 min, the reaction was terminated by heating in a boiling-water bath for 1 min. The protein precipitate was removed by centrifugation and a 0.3-ml portion of the supernatant was assayed for glucosamine-6-phosphate by the method of Levvy and McAllan (3).

Assay of UDP-N-acetylglucosamine 2′-epimerase (EP). The procedure used was based upon the measurement of *N*-acetylmannosamine produced in the reaction mixture using a modified Morgan–Elson procedure as described by Spivak and Roseman (4). The assay mixture contained 2 m*M* UDP-GlcNAc, 200 m*M* Tris-HCl buffer, pH 7.5, 80 m*M* $MgSO_4$, and 0.1 ml enzyme extract in a total volume of 0.25 ml. Incubation was carried out at 37° for 20 min and the reaction was terminated by heating in a boiling-water bath for 2 min. The protein

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itate was removed by centrifugation 0.1-ml aliquot of the supernatant was d in a test tube along with approxi- y 80–100 mg of Dowex-2 acetate (20). Three-tenths milliliter of water was l to increas the volume. The tubes mixed vigorously and the reaction mix- vas allowed to react with the resin for 5 min. The resin was removed by fugation and 0.2 ml of the supernatant sed for the estimation of N-acetylman- nine.

tein assay. The protein content of the 00*g* supernatant was determined by a ied biuret method according to Wolf- *al.* (5) using crystalline bovine albu- s a standard. Enzyme specific activities expressed as nanomoles of glucosa- 6-phosphate formed per milligram of in per hour for AT and as nanomoles acetylmannosamine formed per milli- of protein per hour for EP.

P N-acetylglucosamine assays. The ntrations of hepatic UDP-GlcNAc determined as previously described (1) modification of the procedure of Bates Handschumacher (6). One volume of w/v trichloroacetic acid (TCA) was d to 9 vol of 105,000*g* supernatant. removing acid-insoluble material by ifugation, the supernatant was heated in in a boiling-water bath to hydrolyze -*N*-acetylhexosamines. The solution entrifuged again to remove heat-coag- d material and 0.5 ml of this superna- vas assayed for hexosamine content by dification of the Elson–Morgan reac- as described by Neuhaus and Letzring A standard curve was prepared using samine hydrochloride. The UDP- Ac pool sizes were expressed as nano- s of UDP-GlcNAc per gram of liver.

ay of CMP-N-acetylneuraminic acid. epatic concentrations of CMP-NANA determined on the same TCA and treated supernatants used to assay -GlcNAc pool sizes. The method used MP-NANA measurements was that of lian *et al.* (8). Five-tenths milliliter of CA supernatant was added to 0.1 ml of *M* periodic acid solution, mixed and ed to stand in ice-cold water for 20 After the addition of 1.25 ml of resorcinol reagent, the solution was mixed, placed in an ice bath for 5 min and heated at 100° for 15 min. The reaction mixture was cooled in tap water, 1.25 ml of tertiary butyl alcohol was added and the mixture agitated vigorously. The tubes were placed in a 37° water bath for 3 min and cooled to room temperature, and the absorbance was measured at 630 nm.

Assay of the seromucoid fraction. The seromucoid fraction was separated from serum by the method of Winzler (9) and was expressed in terms of its protein concentration as assayed by the method of Lowry *et al.* (10).

Results. *L-glutamine*: *D-fructose-6-phosphate aminotransferase (AT).* The changes in AT activity following birth are shown in Fig. 1. The average specific activity of the enzyme for adult animals was 47.7 ± 1.3 nmoles/mg of protein/hr. The enzyme activity at 24 hr after birth was one-third of the adult level (17.9 ± 1.1 nmoles/mg/hr). The AT activity continued to increase until about 14 days after birth, at which point it reached 1.7 times the adult value. The peak value was followed by a gradual decrease over the next 2 weeks, gradually returning to the adult level. The specific activity of the enzyme in older adult animals tended to be slightly lower when compared to that of younger adult animals.

UDP-N-acetylglucosamine 2'-epimerase (EP) activity. As can be seen in Fig. 1 the

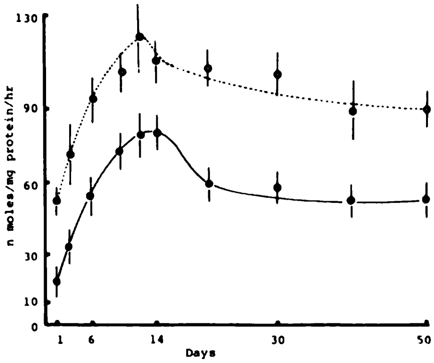

FIG. 1. Changes in specific activities of AT and EP during development. Values are presented as means ± SD. Each point represents the mean of four to six animals. ●——● L-glutamine:D-fructose-6-P aminotransferase (AT). ○----○ UDP-GlcNAc 2' epimerase (EP).

developmental pattern of EP activity was quite similar to that of AT. The average specific activity of the EP for adult animals was 75.0 ± 6.3 nmoles/mg of protein/hr. The enzyme activity at 24 hr after birth was about half the adult value (39.3 ± 4.2 nmoles/mg/hr) and continued to increase to a maximum at about 14 days, at which point it reached 1.5 times the adult level. This was followed by a gradual decrease over the next 2 weeks to adult values.

UDP-N-acetylglucosamine concentration. The developmental changes occurring in the UDP-GlcNAc pool size are shown in Fig. 2. The UDP-GlcNAc pool size in adult livers averaged 436 ± 38 nmoles/g of liver in these experiments. Very high concentrations (963 ± 82 nmoles/g), approximately two times the adult value, were measured at 24 hr after birth. These values then continued to decline slowly over the next 20 days and after that decreased somewhat more sharply toward the adult level.

CMP-NANA concentrations. The developmental changes occurring in hepatic CMP-NANA concentration are shown in Fig. 3. As compared to UDP-GlcNAc, less dramatic changes were observed in the CMP-NANA pool with development. CMP-NANA maintained a fairly constant concentration until about 20 days after birth, decreased sharply between 20 and 30 days and then plateaued for the next 20 days. The CMP-NANA concentration of adult livers averaged 157 ± 13 nmoles/g liver in these experiments.

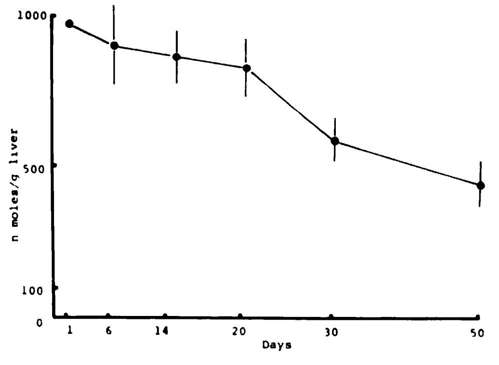

FIG. 2. Changes in hepatic UDP-GlcNAc concentration during development. Assays were carried out on the same 105,000g supernatant used to assay the enzyme activities presented in Fig. 1. Values are presented as means ± SD. Each point represents the mean of four to six animals.

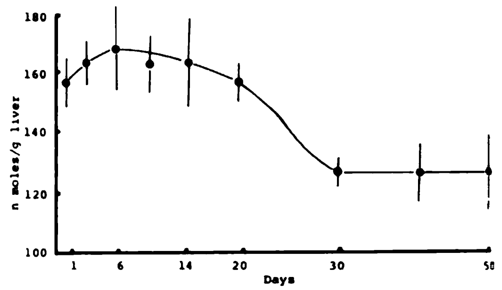

FIG. 3. Changes in hepatic CMP-NANA concentration during development. Assays were carried out on the same 105,000g supernatant used to assay the enzyme activities presented in Fig. 1. Values are presented as means ± SD. Each point represents the mean of four to six animals.

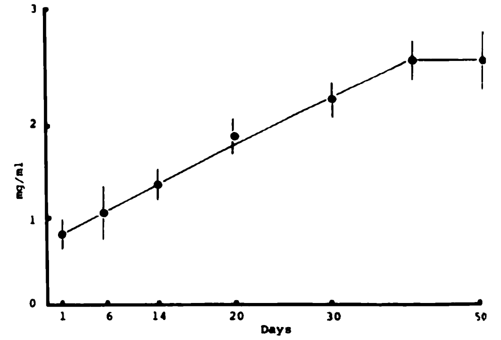

FIG. 4. Changes in serum seromucoid concentration during development. Blood was obtained from the abdominal aorta, and the seromucoid fractions were prepared by the method of Winzler (9). Values are presented as means ± SD. Each point represents the mean of four to six animals.

Seromucoid concentrations. The changes in seromucoid concentration with development are shown in Fig. 4. The average concentration of the seromucoid fraction for adult animals was 2775 ± 86 μg/ml of serum. Much lower concentrations, about one-third of the adult value, were measured at 24 hr after birth. The seromucoid concentration then continued to increase linearly with development to about the 30th day, after which the rate of increase declined to the adult level 50 days after birth.

Discussion. The general pattern of change observed in AT and EP specific activities agreed quite well with that reported by Kikuchi *et al.* (11), with the exception that they

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is to be incorporated into the glycoproteins of the seromucoid fraction, then the instantaneous concentration of these nucleotide sugars would be a function of the rate at which they are being synthesized and transferred to the glycoproteins of the blood plasma. There is, of course, some influence of cell growth and proliferation, but quantitatively this would be minor compared to blood glycoprotein production and secretion.

The adult levels found for CMP-NANA concentrations obtained in these experiments agree well with the values reported by Harms *et al.* (12) for normal liver. Although the method employed actually measured *free* NANA, previous work has shown that almost all of the NANA found in 105,000g supernatants of liver tissue exists as CMP-NANA. Liver supernatants not subjected to hydrolysis show only traces of free NANA.

It is difficult to explain the increase in the older adult animals after the seromucoid levels have come to equilibrium, but may reflect developmental changes in sialyltransferase activity.

Both UDP-GlcNAc and CMP-NANA are known to be feedback inhibitors of the initial enzymes in their respective pathways (13). What effect the changes in the hepatic concentrations of these compounds has on the specific activities of these enzymes is difficult to evaluate, since it is clear that the feedback inhibition exerted can be very complex. Winterburn and Phelps (14) and Miyagi and Tsuiki (15) for example, have shown that the control of AT is not regulated by fluctuation in the UDP-GlcNAc concentrations but rather is due to alterations in the AT:UDP-GlcNAc binding constant evoked by the secondary effectors UTP, G-6-P, and AMP. Similar complex relationship may exist between EP and CMP-NANA. Further investigations involving measurements of the intracellular concentrations of such secondary effectors with development must be carried out before the relationship between enzyme activity and amino sugar nucleotides can be clarified.

Summary. Changes in hepatic hexosamine metabolism and serum seromucoid concentrations during postnatal development were investigated in the rat. A rela-

tively low activity of hepatic L-glutamine: D-fructose-6-phosphate aminotransferase (AT) was observed within 24 hr of birth. This rapidly increased to a maximum at about 2 weeks of age, followed by a decline to adult levels after another 2 weeks. The developmental pattern of hepatic UDP-*N*-acetylglucosamine 2′-epimerase (EP) closely resembled that of AT. It was relatively low in the newborn, increased to a maximum at about 2 weeks and then declined to adult values after another 2 weeks. The serum seromucoid concentration was low in new born rats and then increased gradually with development to adult levels at about 50 days.

The hepatic concentrations of UDP-GlcNAc and CMP-NANA both were relatively high at 24 hr after birth compared to values observed at 50 days. The developmental pattern observed for both over this time period was quite similar and was almost the reciprocal to that for the seromucoid fraction. After 50 days the concentration of UDP-GlcNAc remained constant at adult levels as did that of the seromucoid fraction, but the concentration of CMP-NANA showed a marked increase.

It is suggested that the hepatic concentrations of these amino-sugar nucleotides during development primarily reflect the demand for the hepatic synthesis and secretion of plasma glycoproteins as reflected in the seromucoid fraction.

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# of Converting Enzyme in the Cardiovascular and Adrenal Cortical Responses to (des-Asp¹)-Angiotensin I[1] (39456)

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e C-terminal heptapeptide homolog of ıtensin II, angiotensin III, has been ınstrated to be an active peptide of the -angiotensin-aldosterone system in a ɔer of biologic systems (1). Most nota- as been its steroidogenic effect on the ıal zona glomerulosa in a variety of es (2–4) including man (5). Two path- for angiotensin III formation can be ılated. Classically, angiotensin I, the ɔeptide, is acted upon by converting nes to generate the octapeptide, angi- in II. Subsequently, aminopeptidases ı catalyze the degradation of angioten- [(6) could lead to the formation of ıtensin III. Alternatively, aminopepti- could act initially upon the decapep- angiotensin I, to form the C-terminal peptide, (des-Asp¹)-angiotensin I. Hy- sis of the nonapeptide by converting nes would yield the heptapeptide, an- nsin III (2).

e conversion of synthetic (des-Asp¹)- ıtensin I by purified converting en- s has been demonstrated *in vitro*. The alues and the $V_{\max}/K_m$ ratios favored onapeptide over the decapeptide with enzymes. Therefore, the nonapeptide ıctually a better substrate for porcine ıa and pulmonary converting enzymes was angiotensin I (7).

giotensin III has both steroidogenic ɔressor activity in the intact rat (8). ɔtensin III has been isolated and quan- from the plasma of man, dog, and rat 3). The plasma concentration of angi- in III relative to angiotensin II is quite high in the rat (10). Hence, in the present study, the rat was utilized to compare the relative steroidogenic and pressor effects of (des-Asp¹)-angiotensin I and angiotensin III. In addition, we determined if the effects of the C-terminal nonapeptide of angiotensin I were direct or dependent on cleavage of the nonapeptide by converting enzymes and generation of the active heptapeptide.

Materials and methods. Male Wistar rats (200–300 g) were maintained on low sodium rat chow (4 μEq Na^+/g, General Biochemicals) and 0.5% saline solution *ad libitum* for at least 5 days to insure normal sodium intake. They were housed individually in metabolic cages in a temperature controlled room (20–22°) illuminated between 6:00 AM and 6:00 PM.

Pressor responses. A group of 10 rats was anesthetized with ether. Following bilateral nephrectomy, the iliac artery and femoral vein were cannulated with PE-50 intramedic tubing. The animals were allowed to awaken and stabilize for 1 to 2 hr in restraining cages. Blood pressure was monitored through the arterial cannula attached to a Statham P23D pressure transducer connected to a Brush Mark 220 recorder. Peptides were administered intravenously via a Harvard infusion pump in a 50-μl volume of 5% dextrose in water (D_5W) and flushed with 50 μl of D_5W. (Des-Asp¹)-angiotensin I, angiotensin II, and angiotensin III were administered in random order. One-half hour after administration of the last peptide, a 300 μg/kg bolus injection of converting enzyme inhibitor, *Bothrops jararaca* nonapeptide (Spectrum Medical Industries Lot No. 215865), was given in a volume of 100 μl. Responses to (des-Asp¹)-angiotensin I and angiotensin II were obtained after treatment with the enzyme inhibitor. The angiotensin nona- and heptapeptides were administered only during a time period from 5

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to 20 min after treatment with the *B. jararaca* peptide.

Steroidogenic response. An additional 24 rats were killed by decapitation and the adrenal glands were quickly removed and cleaned. The inner zones and medulla were removed and the remaining capsular layer was minced. The minced tissue was incubated in a Dubnoff metabolic shaker for 15 min at 60 rpm under an atmosphere of 95% O_2/5% CO_2 in Krebs-Ringer bicarbonate buffer containing 0.2% D-glucose, 4% albumin, 0.008% (DNase) (KRBGAD), and 0.2% trypsin. The tissue was washed twice with KRBGAD containing 0.1% trypsin inhibitor, followed by incubation for 20 min at 120 rpm in KRBGAD with 0.1% trypsin inhibitor and 0.04% collagenase. The tissues were then pipetted 75 times with a 1-ml biopipet and reincubated for 10 min. The dissociated cells were filtered through 150-μm^2 nylon mesh and the filtrate centrifuged at 4° for 15 min at 100*g*. The pellet was washed and finally resuspended in 1 ml of KRBGA. The number of viable cells was determined by exclusion staining with nigrosin and counting with a Neubauer hemocytometer.

The cell suspensions were divided into aliquots containing 5×10^4 cells, diluted to 1 ml with KRBGA, and equilibrated for 30 min at 60 rpm in a Dubnoff bath (37°, 95% O_2, 5% CO_2). Steroidogenic compounds to be tested were added in 10-μl volumes after the equilibration period. The same amount of vehicle was added to controls. When utilized, *B. jararaca* nonapeptide was administered 5 min prior to the addition of the angiotensins. The experimental incubation time was 1 hr after addition of steroidogenic peptides.

The LH-20 column purification and aldosterone assay were performed according to the method of Sarstedt *et al.* (11).

Results. Pressor effects. The cardiovascular responses to angiotensin II, (des-Asp^1)-angiotensin I, and angiotensin III are shown in Fig. 1. Both the nonapeptide and the heptapeptide exhibited about one-third of the pressor activity of angiotensin II. There was no statistical difference between the pressor responses to the nonapeptide and angiotensin III.

Pretreatment with the converting enzyme inhibitor (CEI), *B. jararaca* nonapeptide, at a fixed dose of 300 μg/kg (dose required to block the response to an ED_{50} dose of angiotensin I) markedly reduced ($p < 0.01$) the pressor activity of (des-Asp^1)-angiotensin I at all doses administered (Fig. 2). Inhibition of converting enzyme did not alter the pressor response to angiotensin III. Since higher concentrations of CEI were not utilized, residual pressor activity of the nonapeptide may represent either uninhibited converting enzyme activity or the direct intrinsic activity of the angiotensin nonapeptide (or both).

To clearly quantify the intrinsic activity of (des-Asp^1)-angiotensin I on aldosterone

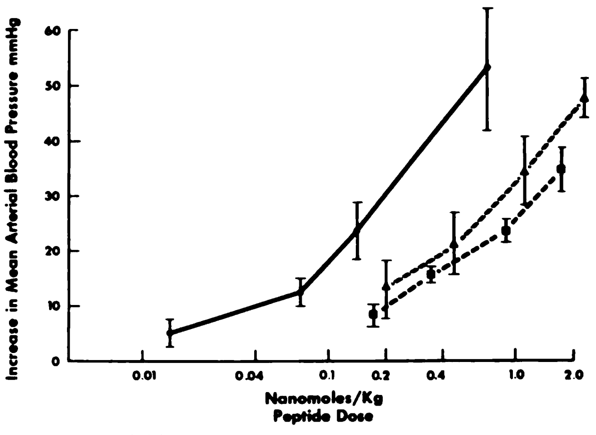
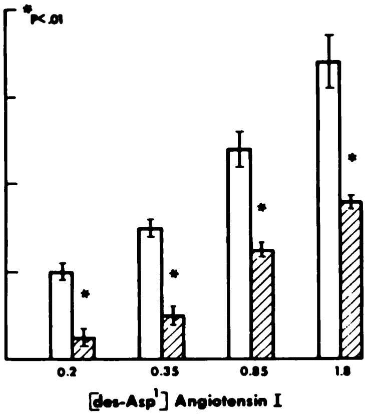


FIG. 1. Increase in mean arterial blood pressure induced by angiotensin II (●——●), angiotensin III (▲- - -▲), and [des-Asp^1]-angiotensin I (■- - -■). Each point represents the mean ± SEM of eight experimental observations.



Inhibition of [des-Asp¹]-angiotensin I-in- pressor responses with 300 μg/kg of *B.* onapeptide (■). Each bar represents the :M of eight observations. The * indicates $p <$

esis, the *in vitro* zona glomerulosa ›ensions were studied. In the rat zona glomerulosa, (des-Asp¹)-angi- had only 0.1% of the steroidogenic f angiotensin III (Fig. 3). After it with CEI, the concentration of P¹)-angiotensin I had to be in- to 1 × 10^{-6} *M* before a significant dosterone ($p < 0.01$) occurred. In ıdies with rat adrenal cell suspen- giotensin I was found to be about ›otent as angiotensin III, and the to the decapeptide was not altered ıtly by treatment with the *B. jara-* apeptide (data not shown). In con- comparable response was obtained 10^{-9} *M* angiotensin III. The weak genic response to (des-Asp¹)-angi- [reflected direct activity since the was not significantly inhibited (*p* ›y 1 × 10^{-6} *M* or higher concentra- CEI. Converting enzyme inhibitor ffect on basal steroidogenesis or on sin III-induced aldosterone biosyn-

sion. It has been established in the angiotensin III induces a steroido- sponse comparable to that of angi- [I and possesses about one-third of sor potency of angiotensin II (8). giotensin III is the product of the al actions of both converting en- nd aminopeptidases, it is theoretically possible that (des-Asp¹)-angiotensin I could be a precursor of angiotensin III. However, this would necessitate the rapid hydrolysis of the nonapeptide by converting enzymes *in vivo*. (Des-Asp¹)-angiotensin I has been shown to be a substrate for porcine-converting enzymes *in vitro* (7). Administration of the nonapeptide in the conscious rat produced pressor changes indistinguishable from those obtained with the heptapeptide. In addition, this pressor response could be significantly attentuated by pretreatment with the CEI, indicative of rapid conversion. Residual pressor effects could be due to intrinsic activity of the nonapeptide or incomplete inhibition of conversion. The latter explanation is more plausible since (des-Asp¹)-angiotensin I has very little direct biologic activity on the isolated rat uterus (12). In the rat, if (des-Asp¹)-angiotensin I is present in the circulation, it will serve as a substrate for coverting enzymes and therefore is a potential precursor of angiotensin III.

These *in vivo* studies provided evidence for the potential alternate pathway for the generation of angiotensin III and stimulated evaluation of (des-Asp¹)-angiotensin I on aldosterone biosynthesis. Sodium deprivation results in renin release, activation of aminopeptidases (6), and increased aldos-

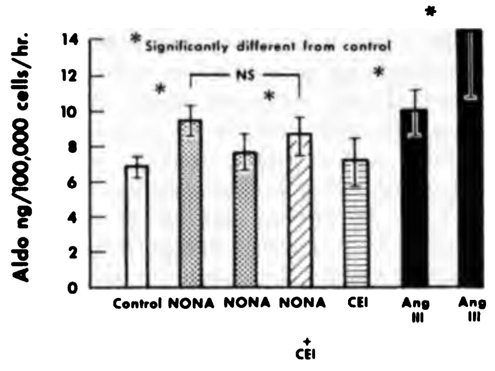


FIG. 3. Steroidogenic actions of [des-Asp¹]-angiotensin I and angiotensin III in rat adrenal cell suspensions. Bars from left to right represent control (□); [des-Asp¹]-angiotensin I 1 × 10^{-6} and 1 × 10^{-7} *M* (▩); [des-Asp¹]-angiotensin I 1 × 10^{-6} *M* plus *B. jararaca* nonapeptide 1 × 10^{-6} *M* (▨); *B. jararaca* nonapeptide 1 × 10^{-6} *M* (■); angiotensin III 1 × 10^{-9} and 1 × 10^{-8} *M* (■). Each bar represents the mean ± SEM of 5-6 observations in different cell preparations. The * indicate $p < 0.05$.

terone biosynthesis which is resistant to blockade by angiotensin II antagonists (11). A role for angiotensin III-induced aldosterone biosynthesis in sodium deficient states is suggested by the finding that subcutaneous administration of (Ile^7)-angiotensin III partially decreased aldosterone biosynthesis (11). The present studies on steroidogenesis were performed *in vitro* so that accurate assessment of the direct steroidogenic activity of (des-Asp^1)-angiotensin I could be obtained. The aldosterone responses of cell suspensions show that (des-Asp^1)-angiotensin I has little capacity to stimulate steroid biosynthesis. However, the increase in aldosterone synthesis obtained with micromolar concentrations of angiotensin nonapeptide (0.1% of the steroidogenic effect of angiotensin III) represents direct activity since it was not inhibited by CEI.

The results of the present investigation support the proposed alternate pathway for the formation of angiotensin III from (des-Asp^1)-angiotensin I. Therefore, angiotensin III may be formed by the C-terminal hydrolysis of (des-Asp^1)-angiotensin I and/or the N-terminal degradation of angiotensin II.

Summary. (Des-Asp^1)-angiotensin I, angiotensin II and III were evaluated for pressor activities in conscious nephrectomized rats and for steroidogenic actions in rat adrenal zona glomerulosa. The pressor effect of this angiotensin nonapeptide was similar to that found with mole-equivalent doses of angiotensin III (one-third as active as angiotensin II) and was significantly attenuated by pretreatment with the *B. jararaca* nonapeptide converting enzyme inhibitor. Hence, (des-Asp^1)-angiotensin I is a substrate for converting enzyme *in vivo*, and the rapid conversion indicates that an alternate pathway for the formation of angiotensin III could exist. (Des-Asp^1)-angiotensin I possessed only 0.1% of the activity of angiotensin III as a steroidogenic agent in cell suspensions of rat adrenal zona glomerulosa. Angiotensin I was a weak steroidogenic agent *in vitro* (1%) and was not blocked by an inhibitor of converting enzyme. Adrenal cells dispersed from the outer zone of the cortex would appear to be devoid of significant converting enzyme activity.

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ulfhydryl Group of Monellin: Its Chemical Reactivity and Importance to the Sweet Taste (39457)

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chemostimulatory protein monellin, protein of molecular weight 10,700, tensely sweet (1-4). A combination ence from amino acid analyses and lusion chromatography shows that cies of minimal molecular weight weet and contains a single cysteine (2). Van der Wel and Loeve (3), r, reported finding no free —SH in tein using Ellman's reagent. Rather, ported that reduction of monellin 5 *M* cysteine or ethanethiol elimi- he sweet taste, and they suggested -SS— bond is involved in its sweet- he tertiary structure does appear to rtant for the sweet taste of monellin

earlier findings that the monellin e contains a single cysteine residue ve (sweet) molecule (2) are, there- variance with the reported presence S— bond. The possibility that mo- ight be a dimer of molecular weight in its sweet-tasting form was ruled In this paper we demonstrate that a ree -SH is present in native monel- that chemical modifications of the ad to a concomitant decrease in ss of the protein.

ials and methods. Iodoacetic acid, ptoethanol (Eastman), high purity e-hydrochloride (Gdn-HCl) (Heico, elaware Water Gap, Pa.), 5,5′- is(2-nitrobenzoic acid) (DTNB), xymercuribenzoate (sodium salt)), cysteine (free base), dithioeryth- dium dodecyl sulfate (Sigma), and e urea (Schwarz-Mann) were used without further purification. Monellin was purified as described previously (1, 5), and solutions were prepared from lyophilized protein held over P_2O_5 *in vacuo* at 4°. Other chemicals were reagent grade.

The —SH content of native, denatured, or carboxymethylated monellin was determined by titration with PHMB using the procedure of Boyer (6) as described by Riordan and Vallee (7). Titrations were performed at room temperature (24-25°) in an initial reaction volume of 4 ml, buffered at either pH 7.0 (0.01 *M* sodium phosphate) or pH 4.5 (sodium acetate). The monellin for the sample in acetate was prepared in 1.5 ml of 0.1 *N* acetic acid and added to the cuvette containing 2.5 ml of 0.33 *M* Na acetate, final pH 4.5. The appropriate denaturant was in the cuvette, and the reference cuvette had only the protein omitted. After addition of each 10-μl aliquot of PHMB (containing approximately 10 nmoles), the absorbance was measured until it reached a constant value. The absorbance at either 250 nm (pH 7.0) or 255 nm (pH 4.5) was measured in 1-cm path length quartz cuvettes with a Zeiss PMQ II spectrophotometer. Protein concentration was adjusted to between 60 and 80 nmoles of monellin per 4 ml, and was determined for each titration from the absorbance at 277 nm (2). The reactivity of the PHMB was confirmed by titration of 2-mercaptoethanol and of dithioerythritol.

Monellin was titrated with DTNB (24°) according to the method of Ellman (8) in either the absence or presence of denaturants. The sample cuvette contained, in a total volume of 3 ml, 150 μmoles of sodium phosphate buffer (pH 8.0), 0.2 μmole of DTNB, and usually about 75 nmoles (0.8 mg) of monellin. The reference cuvette contained the same mixture without protein. The increase in absorbance at 412 nm was

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followed in 1-cm path length quartz cuvettes in the Zeiss PMQ II. As controls, 2-mercaptoethanol or dithioerythritol were titrated similarly in the absence and presence of the denaturants.

Monellin was carboxymethylated with iodoacetate (9) in 0.05 *M* Tris-HCl buffer (pH 8.4), in the absence and presence of 6 *M* Gdn-HCl. Monellin was dissolved in the Tris buffer or the Tris-guanidine solution immediately before use. Carboxymethylation of protein samples of approximately 10 mg was carried out at ambient temperature (26–28°) in a total volume of 2 ml with magnetic stirring for 2 hr. The reaction was initiated by adding the iodoacetate (10 μmoles in 0.1 ml of the buffer). A control sample stirred in buffered Gdn-HCl was included. The pH was maintained between 8.4 and 8.5 by adding small aliquots of 1 *N* NaOH. After 2 hr, the reaction mixture was cooled in ice and dialyzed overnight at 4° against 4 liters of 0.01 *M* sodium phosphate buffer (pH 7.1). During dialysis of the guanidine-containing samples (carboxymethylated and control), small amounts of protein precipitated. This precipitated protein, which amounted to less than 5% of the original samples, was removed by centrifugation and the supernatant solutions were used for the taste assays and —SH titrations. The sedimented material was dissolved in 1.5 ml of 0.1 *N* acetic acid (precipitates were not soluble in neutral solutions) and also analyzed for —SH content, but adequate taste evaluation was difficult because of the high acid concentration relative to the concentration of monellin.

Samples of monellin (1 mg) were treated with 2-mercaptoethanol (7 m*M*, 0.05 *M*, or 0.71 *M*), cysteine (0.5 *M*), or dithioerythritol (0.05 *M*) (10), in either 0.01 *M* sodium phosphate buffer (pH 7.0), or 0.01 *N* acetic acid (pH 2.9). Thiols were present in 15- to 1500-fold molar excess over the protein. Reductions were carried out at room temperature (24°) for 1 hr (cysteine) or 18 and 24 hr (mercaptoethanol or dithioerythritol).

For air-exposure of monellin, samples were dissolved (5 mg/ml) in either 0.01 *M* sodium phosphate buffer (pH 7.0) (control), 6 *M* Gdn-HCl, or 6 *M* Gdn-HCl containing 0.14 *M* mercaptoethanol. The latter two solutions were prepared in the phosphate buffer and titrated to pH 7.0 with NaOH. The thiol-containing sample was flushed with nitrogen and kept covered with parafilm, while the other two samples were left exposed to the air. The samples were magnetically stirred for 2 hr at ambient temperature (24–26°). The two samples left exposed to the air were then dialyzed overnight at 4° against 2 liters of 0.1 *N* acetic acid; the thiol-containing sample was dialyzed against 1 liter of 0.1 *N* acetic acid that contained 14 m*M* mercaptoethanol. The dialyzed samples were assayed for sweetness and protein concentration.

Protein concentration was estimated from the absorbance of monellin at 277 nm ($\epsilon_{277\,\mathrm{nm}} = 1.47 \times 10^4\ M^{-1}\ \mathrm{cm}^{-1}$ [2]). Sweetener activity was estimated as described previously (1).

Results. Content of free —SH. Reaction of native monellin with PHMB was very slow, demonstrating that the —SH is relatively inaccessible. Complete reaction of each aliquot of added PHMB (approximately 10 nmoles) required 30 min or longer (Table I), in contrast to the essentially instantaneous reaction of identical aliquots of PHMB with small molecule thiols (e.g., mercaptoethanol). At either pH 7.0 or pH 4.6 the presence of 1 mole of titratable —SH per mole of protein was found, confirming the presence of one cysteine residue that was established by amino acid analyses (2). Monellin titrated with PHMB in the presence of each of three common protein denaturants showed close to 1 titratable —SH per molecule of monellin (Table I), and in contrast to the slow reaction with native monellin, denatured monellin reacted very rapidly with each aliquot of added PHMB. This clearly demonstrated the increased accessibility of the —SH.

The presence of a single titratable —SH in monellin was also confirmed by titration with DTNB (Ellman's reagent) (Table II). As with PHMB, DTNB reacted extremely slowly with native monellin; complete reaction would require more than 25 hr, compared with 2 to 5 min for complete reaction of DTNB with mercaptoethanol or dithioerythritol. The long time period required for the —SH of native monellin to react ex-

TABLE I. SULFHYDRYL CONTENT OF MONELLIN BY PHMB TITRATION.

Titration conditions[a]	Nanomoles of monellin	Nanomoles of −SH	Moles −SH/ mole of monellin	Reaction time[b]
Sodium phosphate, pH 7.0	76.0	76.0	1.00	30 min
Sodium acetate, pH 4.5	49.6	46.5	0.94	45 min
1% Sodium dodecyl sulfate	70.3	58.5	0.83	30 sec
6 *M* Gdn-HCl	67.8	44.0	0.65	30 sec
8 *M* Urea	69.0	51.5	0.75	30 sec

[a] Titrations in denaturants were carried out in the phosphate buffer (pH 7.0) as described under Materials and ethods. (The first sample is also the first sample in Table III.)

[b] Reaction time for the first two samples, without denaturant, is the minimum time required for each aliquot of ded PHMB to react (except for the initial addition, which was somewhat more rapid). The reaction time dicated for each of the three samples in denaturants is the maximum time required for complete reaction of each ded aliquot. For these samples, reaction of each aliquot of PHMB was essentially complete in the time required mix the sample and place it into the spectrophotometer.

TABLE II. SULFHYDRYL CONTENT OF MONELLIN BY DTNB TITRATION.

Titration conditions[a]	Nanomoles of monellin	Nanomoles of −SH	Moles −SH/ mole of monellin	Reaction time[b]
0.05 *M* Na phosphate, pH 8.0	70.4	52.5	0.74	>25 hr
1% Sodium dodecyl sulfate	70.4	62.4	0.89	10 min
5.7 *M* Gdn-HCl	70.4	48.2[c]	0.69[c]	10 min
7.6 *M* Urea	70.4	58.6	0.83	50 min

[a] Titrations were carried out in the phosphate buffer (pH 8.0) as described under Materials and Methods. The iginal denaturant concentrations for the last two samples were 6 *M* (Gdn-HCl) and 8 *M* (urea), but addition of quots of protein (in deionized water) and DTNB (in 0.05 *M* sodium phosphate buffer, pH 7.0) resulted in the al concentrations indicated. (The titration curves for the samples in denaturants are shown in Fig. 1.)

[b] The reaction time is the approximate time to reach the maximal absorbance at 412 nm (see Fig. 1). Even after hr the absorbance for the first sample (native) had not reached a maximum.

[c] DTNB titrations proved to be less reliable in the presence of Gdn-HCl because the colored reaction product s unstable (see Fig. 1 and inset).

ains the absence of —SH in monellin re- rted by van der Wel and Loeve (3). In the esence of a denaturant, the —SH group of onellin reacted much more rapidly (Table , Fig. 1), and titration with DTNB showed e presence of approximately 1 —SH per olecule (Table II). DTNB titrations in dn-HCl are less reliable than in the other naturants because the colored reaction oduct is unstable in Gdn-HCl (Fig. 1). he maximal absorbance reached before the lor began to fade was usually 70 to 80% theoretical. This chromophore instability, hich was encountered both with monellin d small molecule thiols (inset to Fig. 1), s been reported previously (11) and is ubtless due to the Gdn-HCl or a trace impurity in it. For example, monellin that as first denatured in Gdn-HCl and then alyzed, subsequently showed a normal ti- ation in the presence of dodecyl sulfate.

Monellin no longer tastes sweet after complete titration with either PHMB or DTNB. When native monellin had been titrated, the loss of sweetness was not reversed by dialysis against phosphate buffer (pH 7.0) containing a large excess (10^4 to 10^6 molar excess) of either mercaptoethanol or dithioerythritol. However, full reversal was achieved under the following conditions. Monellin in the presence of 8 *M* urea was inactivated by a 20-fold molar excess of PHMB; the protein was then dialyzed first against 0.1 *N* acetic acid containing 7 m*M* mercaptoethanol, then against large volumes of 0.1 *N* acetic acid alone, and finally against 0.01 *M* sodium phosphate buffer (pH 7.0). The recovered protein tasted sweet. (The final two dialysis steps are probably not essential for recovery of sweetness, but were included in this particular experiment because of other studies that were carried out on the protein sample.)

Carboxymethylation of monellin. Treatment of native monellin with iodoacetate for 2 hr resulted in only a slight decrease in PHMB-titratable —SH content (Table III). The retention of 88% of the titratable —SH corresponded quite well with the 82% retention of sweetness. Monellin retains its sweet taste following treatment with 6 *M* Gdn-HCl at acid pH, although some loss in sweetness occurs after denaturation at neutral or slightly alkaline pH (5). Advantage was taken of the latter observation. Monellin that was carboxymethylated in the presence of Gdn-HCl at alkaline pH was completely devoid both of titratable —SH and of sweetness (Table III), while the control sample incubated in Gdn-HCl had retained 52% of the sweetness, closely paralleling the 60% retention of titratable —SH.

Reduction and air-oxidation. It was recently reported that reduction of monellin with thiols resulted in loss of sweetness (3). This has not been confirmed. Monellin maintained at neutral pH in the presence of 0.5 *M* cysteine (1 hr), 7 m*M* – 0.71 *M* mercaptoethanol (18 hr), or 0.05 *M* dithioerythritol (24 hr) retained full sweetness activity. Exposure to the latter two reducing agents (0.05 *M*) at low pH for 24 hr was also ineffective in decreasing sweetness.

The somewhat low —SH values determined in the presence of denaturants are possibly due to oxidation of the —SH (5).

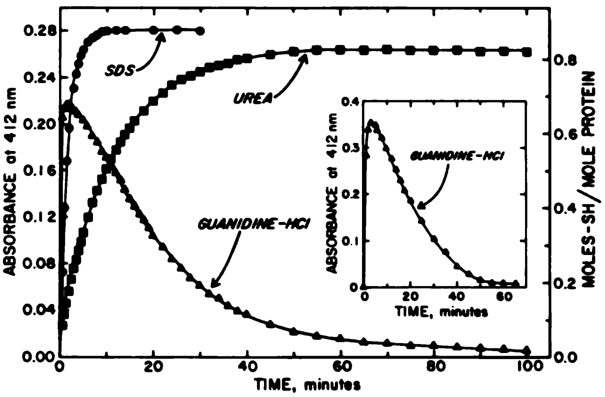

FIG. 1. DTNB titration of denatured monellin. A stock solution of monellin was prepared in deionized water and aliquots (0.16 ml containing 70.4 nmoles) were titrated in the presence of the indicated denaturants in 0.05 *M* sodium phosphate buffer (pH 8.0). The concentrations of denaturants were 1% (w/v) sodium dodecyl sulfate, 5.7 *M* Gdn-HCl, and 7.6 *M* urea. These are the samples shown in Table II. The *inset* shows the titration curve for 2-mercaptoethanol (100 nmoles) in 6 *M* Gdn-HCl.

TABLE III. CARBOXYMETHYLATION OF MONELLIN.

Treatment	Sweetener activity (units/mg)	Percent of native	Theoretical –SH[a] (nmoles)	Observed –SH[b] (nmoles)	Percent of native
Native	174	100	76.0	76.0	100
Carboxymethylated in buffer, pH 8.4	142	82	65.6	57.5	87.5
Buffered 6 *M* Gdn-HCl, pH 8.4	90[c]	52	74.0	44.5[c]	60.2
Carboxymethylated in buffered 6 *M* Gdn-HCl, pH 8.4	0[c]	0	80.8	0[c]	0

[a] Calculated from the absorbance at 277 nm (2) and the presence of 1 cysteine residue per 10,700 g of protein.

[b] Determination of –SH was by PHMB titration. Each 10 μl aliquot of added PHMB (approximately 10 nmoles) was allowed to react for at least 30 min.

[c] Values are for the soluble protein remaining after the small amounts of precipitates (5%) were removed by centrifugation (see Materials and Methods). The dissolved (in 0.1 *N* acetic acid) precipitates were not sweet; slight sweetness could have been masked by the sour taste of the acetic acid. PHMB titration of these dissolved precipitates revealed no titratable –SH.

TABLE IV. TIME-DEPENDENT LOSS OF TITRATABLE SULFHYDRYL OF DENATURED MONELLIN[a].

Conditions	Incubation time	Nanomoles of monellin	Nanomoles of −SH[b]	Moles -SH/mole of monellin
6 *M* Gdn-HCl	15 min	42.2	27.1	0.64
8 *M* Urea	15 min	42.3	33.3	0.79
6 *M* Gdn-HCl	2 hr	43.7	26.2	0.60
8 *M* Urea	2 hr	42.9	16.0	0.37
6 *M* Gdn-HCl	6 hr	39.3	22.2	0.56
8 *M* Urea	6 hr	42.9	5.6	0.13
6 *M* Gdn-HCl	24.5 hr	43.5	13.3	0.31
8 *M* Urea	24.5 hr	39.2	0	0

Samples (nominally 5 mg/ml) were prepared in 0.05 *M* sodium phosphate buffer (pH 7.0) that contained
:r 6 *M* Gd-HCl or 8 *M* urea, and kept, without stirring, throughout the experiment at room temperature
).
For determining −SH content, aliquots (0.1 ml) were withdrawn at the times indicated and titrated with
IB in phosphate (pH 8.0) which also contained the indicated denaturant. Not more than 30 min was required
naximal color development and in most cases the time required was much less (approximately 2.5 min).
ıes for samples titrated in Gdn-HCl are undoubtedly low because of interference of the Gdn-HCl with color
:lopment (see Fig. 1 and inset). Calculations are based on the maximal absorbance reached.

results in Table IV support this inter-
tation, where titratable —SH decreased
ı time in the presence of either denatur-
. To further examine the possibility of a
e relationship between free —SH and
sweetness of monellin, the protein was
osed to oxidation by air under conditions
ely paralleling those of the carboxy-
hylation experiments in Table III. Mo-
in stirred in 6 *M* Gdn-HCl to expose it
ir lost 51% of its sweetness in 2 hr (thus,
ıparing closely with the loss of 48% in
experiment in Table III); in the presence
).14 *M* mercaptoethanol the loss was
24%.

iscusson. The molecular basis for the
et taste of the chemostimulatory protein
ıellin is only beginning to be under-
ıd. The evidence available previously is
sistent with the sweet taste requiring the
ct native protein (4), and recent evi-
ce using denaturants (5) strengthens the
ı that the tertiary structure is important
sweetness.

mino acid analyses (2) showed that a
le cysteine residue is present in the ac-
(sweet) molecule of monellin of molec-
weight 10,700. In contrast, van der Wel
Loeve (3) reported that maintenance of
iary structure was aided by the presence
ı —SS— bond. This was based on the
ırted absence of free —SH and the loss
weetness upon reduction of the protein
hiols. The results of the present study
:radict those of van der Wel and Loeve (3) and point to an important role of the —SH group, not an —SS—, in allowing maintenance of the sweet taste of monellin.

Determination of free —SH by titration with either PHMB or DTNB (Ellman's reagent) gave values approaching 1 mole of —SH per mole (10,700 g) of monellin (Tables I, II). The absence of —SH reported by van der Wel and Loeve (3), who used Ellman's reagent, is readily explained by our finding that the —SH in the native protein is relatively inaccessible to titration. The accessibility of the —SH of monellin to titration increases dramatically in the presence of protein denaturants. The inaccessibility of the —SH in native monellin was also evident when carboxymethylation was attempted, while complete reaction was achieved in the presence of 6 *M* Gdn-HCl. Inaccessibility of —SH to various chemical modifications has been reported for certain other proteins (e.g., 11–13).

The —SH appears to be essential for the sweet taste of monellin, a conclusion supported by several lines of evidence. Monellin is completely inactive (not sweet) after titration with PHMB or DTNB, or when carboxymethylated. Loss of sweetness parallels loss of titratable —SH, and mercaptoethanol helps to protect against loss of sweetness. Inactivation by PHMB is reversed by removing the mercurial with excess mercaptoethanol under appropriate conditions, an observation that also offers the strongest evidence to date that the protein itself tastes sweet. Despite the critical role of the —SH for the sweet taste of monellin, there is no evidence for a direct role of the —SH in eliciting sweetness by interacting with a binding site on taste receptor cells (14), and this type of interaction seems unlikely because the —SH in the native monellin is buried. We postulate that the —SH of monellin must remain unaltered in order for the protein to maintain its tertiary structure and hence its sweet taste.

Summary. The presence of a single cysteine in the sweet-tasting protein monellin was confirmed by titrations with *p*-hydroxymercuribenzoate (PHMB) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The sulfhydryl group in native monellin reacts very slowly with each of these reagents, indicating that the sulfhydryl is relatively inaccessible. In the presence of either 6 *M* guanidine-HCl, 8 *M* urea, or 1% sodium dodecyl sulfate, the rate of reaction of the sulfhydryl group with titrant is dramatically increased. Under a variety of conditions, the presence of 1 mole of sulfhydryl per mole of protein (of molecular weight 10,700) was found.

Reaction of the sulfhydryl by titration with PHMB or DTNB leads to loss of sweetness. The free sulfhydryl is also lost by carboxymethylation of monellin in the presence of guanidine-HCl, yielding a protein that is not sweet. Exposure to air in the presence of denaturant leads to a decrease in the sweetness of monellin. Sweetness of the PHMB-reacted monellin can be recovered upon treatment of the protein with mercaptoethanol, and the partial loss of sweetness that occurs with air exposure is lessened in the presence of mercaptoethanol. It is postulated that alteration of the single sulfhydryl group of monellin leads to a change in the tertiary structure of the protein and hence its sweet taste.

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cts of Penicillin G and Cephalothin on Platelet Function *in Vivo* (39458)

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llin G and related antibiotics have wn by a number of investigators to platelet reactions *in vitro* (1-5). tibiotics have also been reported to cessive bleeding in patients, and to the bleeding times in patients and rs receiving high doses of these , 3-7).

vious studies ((2), and Cazenave *et* J. Haematol., in press) we showed icillin G, carbenicillin, and cephalo- ibit the adherence of platelets to a -coated surface and to the damaged rface of rabbits. We also found that G and cephalothin became associ- h the platelets and that their inhibi- cts *in vitro* persisted after the plate- washed and resuspended in fresh

ugh the antibiotics have been shown platelet function *in vitro*, it has not tablished whether these antibiotics the bleeding time *in vivo* by affect- vessel wall, the plasma proteins, or elets. To investigate this question, treated platelets with penicillin G lothin *in vitro*, washed the platelets, ded them in fresh media, reinfused to thrombocytopenic rabbits, and d their ability to shorten the pro- leeding times of these animals. We o studied the effect of pretreatment it platelets with penicillin G, on survival time upon infusion into rab-

ials and methods. *Reagents*. Penicil- USP sodium, 1650 units/mg) was l from General Biochemicals, Cha- s, Ohio). Cephalothin (Keflin) was Dr. R. S. Dolman, Eli Lilly and Co.), Toronto, Ontario. The antibiot- dissolved in water at a concentra- 150 m*M*. Dilutions to the required ations were made with modified Tyrode solution (no calcium or magnesium); the pH was adjusted to 7.35 and the osmolarity to 290 mOsm. Fresh solutions were prepared immediately before each experiment.

Preparation of suspensions of washed platelets labeled with ^{51}Cr. Suspensions of twice-washed platelets from rabbits were prepared in Tyrode solution containing 0.35% bovine albumin (8). They were labeled with ^{51}Cr in the first washing fluid (2). For the bleeding time studies, the platelets were incubated for 30 min at 22° with 1.5 m*M* (880 units/ml) penicillin G or 1.5 m*M* (0.606 mg/ml) cephalothin before being centrifuged and resuspended in fresh medium at a platelet count of 2,000,000/mm^3 for infusion. For the platelet survival studies, the washed platelets were incubated for 20 min at 37° with 15 m*M* (8800 units/ml) penicillin G and then resuspended in fresh medium at a platelet count of 3,000,000/mm^3 before injection into the rabbits.

Platelet survival. The *in vivo* survival of washed rabbit platelets treated with penicillin G was measured by a modification of the method of Reimers *et al*. (9) using platelet suspensions labeled with ^{51}Cr. Control experiments were done with platelets that had not been treated with the antibiotic, but had been subjected to the same washing and resuspending procedures. The radioactivity of samples of whole blood was determined by the method of Aster and Jandl (10).

Preparation of antiserum to rabbit platelets. The antiserum was raised in sheep and was kindly provided by Dr. J. Gauldie, Department of Pathology, McMaster University, Hamilton, Ontario, Canada. Approximately 200 ml of rabbit blood, pooled from six animals, was collected into 2% EDTA-0.33% saline (9 parts of blood to 1 part anticoagulant). Platelet-rich plasma was centrifuged at 2300*g* for 15 min and the

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platelet pellet was resuspended in 5 ml of 0.85% saline at a platelet count of 5,000,000/mm³. The platelets were disrupted by sonication and the sonicate was stored at −20° in 0.1-ml aliquots. For injection, an aliquot was thawed, mixed with 0.1 ml of complete Freund's adjuvant, and injected subcutaneously into a sheep; this was repeated once a week for 6 weeks. At 7 weeks, the sheep was bled and the serum was prepared, heated for 30 min at 56° to destroy complement, and stored at −20°. The presence of antiplatelet antibody was confirmed by the formation of a precipitin line against a sonicated rabbit platelet suspension on an Ouchterlony plate.

Production of thrombocytopenia. Thrombocytopenia in rabbits was produced by whole body irradiation with a cesium source (930 rad, 465 rad on each side for 14.5 min). During irradiation, both sides of the necks of the rabbits were shielded with a lead ribbon to prevent radiation damage to the blood vessels. The rabbits were given injections of 1 ml penicillin-streptomycin (Derapen-C, with dihydrostreptomycin, Ayerst Laboratories, Montreal, Canada) 24 and 48 hr after irradiation. Seven days later the rabbits received an intravenous injection of 0.3 ml of sheep antiserum to rabbit platelets, 12 to 16 hr before the bleeding time studies were to be done.

Platelet counts and bleeding time determinations. One hour before the bleeding time studies, samples of blood were taken from the ear veins of the thrombocytopenic rabbits and the platelets were counted by the method of Brecher and Cronkite (11). Platelets that had been pretreated with penicillin G or cephalothin (or control platelets) were then injected intravenously into the thrombocytopenic rabbits. Each rabbit received 10^{10} platelets in a volume of 5 ml (the weights of the rabbits were similar (mean ± SEM, 2527 ± 47 g)). Thirty minutes later the rabbits were anesthetized with sodium pentobarbital, the jugular veins were exposed and each was arranged above a small trough. Blood samples were taken by cardiac puncture for platelet counts and for measurement of the percentage recovery of ^{51}Cr-labeled platelets injected. Then a puncture wound was made in a jugular vein with a 23 gauge needle. The wound site was observed until bleeding ceased and the time was recorded. To prevent accumulation of blood at the wound site, a flow of 0.85% saline was directed into the trough below the exposed jugular vein. The bleeding time was then measured again in the same way by puncturing the other jugular vein.

Statistical considerations. In experiments with thrombocytopenic rabbits the relation between the platelet count and the bleeding time has been found to resemble an exponential function (Hirsh, J., and Senyi, A. F., unpublished observation). Therefore, the bleeding time values were transformed to logarithms before determining the mean for each experiment. The significance between any two of the three means was determined by a two-way analysis of variance. This analysis also provides the best common estimate of the standard deviation. This was then used to develop the 99% confidence intervals for each of the two treatment means and the control mean. The calculated means and confidence intervals from the transformed data were then transformed back to the original scale.

Results. Platelet survival. Pretreatment of platelets with penicillin G had no apparent effect on their survival *in vivo* (Fig. 1).

Bleeding times. The range of bleeding

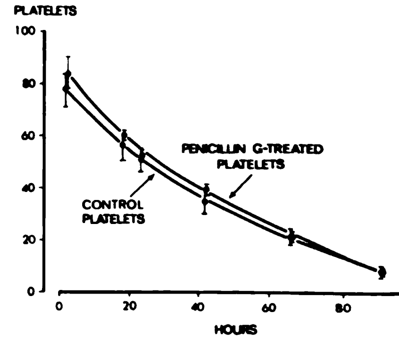

FIG. 1. The effect of penicillin G on survival of control washed rabbit platelets and platelets that had been incubated in vitro with penicillin G (15 m*M* for 20 min at 37°) and resuspended in fresh medium before infusion into the rabbits. Platelets were labeled *in vitro* with ^{51}Cr before penicillin G treatment. Means and standard errors of the means are shown. Four rabbits in each group.

' 41 untreated rabbits (mean platelet SEM, 414,000 ± 12,900/mm^3) was 80 sec [geometric mean and (99% ıce intervals), 100 (73-136)sec]. g time from puncture wounds in the veins of thrombocytopenic rabbits .telet counts of 4000 to 16,000/mm^3 ± SEM, 8000 ± 130/mm^3) ranged ı4 to 1220 sec [geometric mean and :onfidence intervals), 1012 (934- ɜc]; these values were determined in ıte group of 12 rabbits that did not injections of platelets. Platelets ıad not been treated with an anti- ıortened the bleeding time of throm- enic rabbits to within the range of ; times of untreated rabbits with platelet counts (Table I). Although elets that had been pretreated with n G or cephalothin did shorten the ; time from the puncture wounds in .lar veins, they were much less effec- ı the control platelets which had not ɜated with an antibiotic (Table I). n of platelets pretreated with peni- shortened the bleeding time more jection of cephalothin pretreated ;. The observed difference between of the three means was statistically ignificant ($p < 0.001$).

ıean platelet counts of the 21 throm- enic rabbits used for studies with the ic-treated platelets are shown in Ta- Гhe mean platelet counts and the ıge recovery of ^{51}Cr-labeled plate- ıre the bleeding time determinations shown. In no instance was there a statistically significant difference ($p < 0.5$) between the three groups of animals; thus, the effect of the antibiotics on the bleeding time cannot be attributed to differences in platelet counts or platelet recovery.

Discussion. Pretreatment of rabbit platelets with a high concentration of penicillin G *in vitro* did not affect their recovery or survival *in vivo* following reinjection.

The concentration of the antibiotics to which the platelets were initially exposed was much greater than the concentrations usually achieved in man (0.03 to 12 units of penicillin/ml). However, maximal serum concentrations of 1000 to 2000 units/ml have been reported in man following intravenous administration of 20 to 100 million units of penicillin G (12).

The diminished ability of platelets preincubated with penicillin G or cephalothin to shorten the bleeding time of thrombocytopenic rabbits is in keeping with our observations from earlier studies of the effects of these antibiotics on platelet function (Cazenave *et al.*, Brit. J. Haematol., in press). We found that platelets incubated with penicillin G or cephalothin, washed and resuspended in fresh medium responded less strongly to aggregating and release-inducing agents (ADP, collagen, or thrombin) than control platelets. It seems likely that the antibiotics became bound to the platelets. Thus, it is not surprising that platelet function *in vivo* is inhibited by preincubation of the platelets with the antibitoics *in vitro*. The impairment of the ability of platelets to adhere to subendothelial structures and to

.E I. BLEEDING TIMES[a] OF THROMBOCYTOPENIC RABBITS GIVEN PLATELETS PREINCUBATED IN VITRO WITH PENICILLIN G, CEPHALOTHIN, OR NEITHER ANTIBIOTIC.

tic used for ıtment of ıtelets	Number of determinations	Mean platelet count[b] (number/mm^3) Before infusion	Before bleeding time determinations	Percent of recovery of ^{51}Cr-labeled platelets[b]	Bleeding time[c] (sec) mean (confidence interval)
ntrol)	14	4600 ± 600	62,700 ± 4700	68 ± 9	119 (98-144)
G 1.5 m*M*	14	4000 ± 500	56,600 ± 6900	69 ± 8	375 (309-455)
ıin 1.5 m*M*	14	4600 ± 300	55,100 ± 6200	65 ± 4	616 (507-748)

ling times of rabbits that had been made thrombocytopenic in this way and were not given platelet ranged from 764 to 1220 sec (see text).

ı ± SEM.

ıetric mean and 99% confidence intervals. Difference between any two of the three treatment means (*p* two-way analysis of variance).

react to aggregating agents would account for the observation that the antibiotic-treated platelets were much less effective than control platelets in shortening the bleeding times of thrombocytopenic rabbits.

Thus, the effects of penicillin G and related antibiotics on the bleeding times of patients (1, 5–7) and volunteers (3, 4) given high doses of these drugs is, at least in part, attributable to the inhibitory effects of the antibiotics on the platelets. Our results, however, do not exclude the possibilities that the antibiotics also affect the vessel wall and/or the plasma proteins.

Our results support the suggestion of Brown *et al.* (4) that "patients with underlying hemostatic defects, especially patients with thrombocytopenia, may be at an increased risk of hemorrhage" when penicillin G and related antibiotics are administered in high dosages.

We wish to acknowledge the assistance of Professor M. Gent, Department of Clinical Epidemiology and Biostatistics, McMaster University, in the statistical evaluation of the data.

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Characterization of Extracellular Particles Released from Continuous Cell Cultures Derived from Human Leukemia[1] (39459)

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Several studies reported oncornavirus-like particles in media taken from cultured cells derived from patients with malignant hemopoietic and lymphoid disorders (1–4). In contrast, particles with properties unlike oncornaviruses were detected in culture medium of cells derived from patients with Hodgkin's Disease (5, 6), adenocarcinoma (7), and the plasma of patients with chronic lymphocytic leukemia (8).

Barker *et al.* reported that continuous cell lines (Z-597, JIII) derived from human leukemias contained cytoplasmic particles with a density of 1.18 g/cm^3 in sucrose and a DNA polymerase, inhibitable by RNase (9). These contained RNA, DNA, and a covalently linked RNA-DNA complex (10).

The present report describes the detection and characterization of extracellular particles released into the medium of cultured JIII cells, which are distinctly unlike oncornavirions.

Materials and methods. Cell lines. The JIII cell line was derived from peripheral blood of a patient with monocytic leukemia and obtained from American Type Culture Collection Cell Repository, Rockville, Md. The Z-597 cell line was established by Dr. William Murphy, University of Michigan, Ann Arbor from peripheral blood of a patient with granulocytic leukemia. Cells were grown on a glass substrate at 37° with a mixture of 1× Eagle's minimal essential medium (11) and Hank's balanced salt solution (12) which contained 15% fetal calf serum (Flow Laboratories, Rockville, Md.) and antibiotics. Cells were passaged at 5–7 day intervals. Cell lines were free of Mycoplasma as shown by failure to culture aerobically and anaerobically the organisms from partially disrupted cells and culture medium, and by electron microscopic examination of cell cultures.

Processing of particles from tissue culture fluid. Tissue culture fluid was removed from cell monolayers and centrifuged twice at 1000*g* for 30 min at 4°. The supernatant was centrifuged at 55,000*g* in a Beckman Type-30 rotor for 1 hr at 4°. Pellets were resuspended in 0.5 ml of 0.1 *M* Tris-HCl (pH 8.3)–0.1 *M* EGTA and centrifuged to equilibrium in a 12–60% sucrose gradient for 18 hr at 90,000*g*. An aliquot from each fraction collected from the tube was assayed for either DNA polymerase activity or acid-insoluble radioactivity.

Biochemical procedures. Cells grown to a confluent monolayer were labeled with radioactive isotopes by changing the medium to 1× Eagle's with 2% fetal serum and adding either 0.2 μCi/ml of [*methyl*-^{3}H]thymidine (sp act 25 Ci/mmole) or [5-^{3}H]uridine (sp act 21 Ci/mmole) (Nuclear Dynamics). After 24 hr the medium was harvested and processed for particles.

DNA polymerase assays were done as described previously (9). Acid-insoluble radioactivity measurements were performed as described by Barker *et al.* (10).

Electron microscopy. Preparations were placed on Formvar carbon-coated copper grids, stained with 2% phosphotungstic acid, and examined with an AEI Corinth 275 electron microscope.

Immunological procedures. Particles processed from the culture medium were mixed with an equal volume of complete Freund's adjuvant and injected into the footpads of rabbits. Booster injections with a equal volume of Freund's adjuvant were given subcutaneously.

Globulins from the preimmune and immune rabbit serum were conjugated with fluorescein isothiocyanate (Baltimore Biological Laboratory, Cockeysville, Md.) by

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the method of Riggs *et al.* (13). All conjugates were absorbed with a solid matrix of cross linked bovine serum albumin in order to remove antibodies possibly elicited against the albumin fraction of fetal calf serum.

Cells to be examined for extracellular antigens were grown in Leighton tubes with coverslips, fixed in acetone for 10 min, and then tested with either the direct or indirect fluorescent antibody method. Unfixed cells were also tested for surface antigens using the direct fluorescent antibody method (equal volumes of cells and conjugate were incubated).

Result. Release of particles from JIII cells with RNA, DNA, and DNA polymerase. A pellet, sedimented by differential centrifugation from medium of JIII cell cultures, was resuspended and centrifuged to equilibrium in a sucrose gradient. Fractions of the gradient were assayed for DNA polymerase activity with endogenous nucleic acid templates and primers. A peak of enzyme activity was detected in the 1.18–1.22 g/cm^3 region of the gradient (Fig. 1). Prior to collection of the fractions, an optically visible band was observed in the gradient corresponding in position to the polymerase activity.

In another series of experiments, JIII cell cultures were labeled with either [^{3}H]thymidine or [^{3}H]uridine. The culture fluids were processed and centrifuged as described above, whereupon peaks of [^{3}H]thymidine or [^{3}H]uridine radioactivity were found in the 1.18–1.22 density region of the gradient. Thus, the RNA, DNA, and polymerase were coincident with the optically visible band of the gradient (Fig. 1).

Medium from Z-597 cell cultures also yielded particles similar to JIII cultures in density, DNA polymerase and [^{3}H]uridine incorporation. Since such particles could not be recovered from medium of normal skin fibroblast cultures or culture medium incubated without cells, it is concluded that the particles in the media of JIII and Z-597 cultures were released from the cells in those cultures.

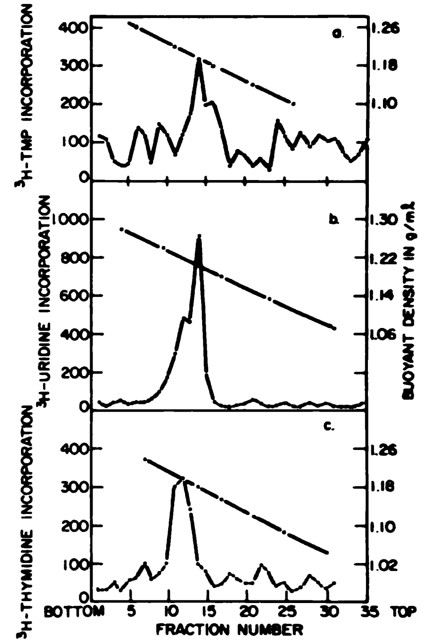


FIG. 1. Buoyant density of JIII particles determined by isopycnic gradient centrifugation. Cell cultures were either (a) unlabeled, (b) labeled with 0.2 μCi of [^{3}H]uridine or (c) labeled with 0.2 μCi/ml of [^{3}H]thymidine for 24 hr. Pellets from the medium of JIII cultures were resuspended in 0.5 ml of 0.1 *M* Tris-HCl (pH 8.3)–0.01 *M* EGTA and centrifuged to equilibrium in a 12–60% sucrose gradient at 90,000*g* for 18 hr at 4°. Fractions (0.45 ml) were collected from the bottom of the gradients (a) 25-μl aliquots of each from the unlabeled gradient were assayed for DNA polymerase activity and expressed as ^{3}H-TMP incorporation; (b) and (c) 25-μl aliquots were assayed for acid insoluble radioactivity.

Properties of the DNA polymerase activity. The DNA polymerase associated with JIII particles was assayed with varying concentrations (0.005–0.200%) of Nonidet, a nonionic detergent. The maximally effective concentration 0.1% increased the enzyme activity over sixfold (Table I). This stimulatory effect suggests that access of the enzyme to components of the assay mixture is limited by lipid.

Four preparations of JIII particles were assayed for DNA polymerase after pretreatment with RNase, with and without an optimal concentration of Nonidet. RNase produced minimal inhibition of DNA polymerase in absence of Nonidet. In its presence inhibition was increased, but only partially in the range of 8–46% (Table II). The results indicate some function of the RNA in the particle on its DNA synthetic activity.

JIII particles, which synthesize DNA with endogenous templates and primers, were tested for capacity to use exogenous RNA and DNA. With exogenous heated calf-thymus DNA, the DNA synthesis of the particles was fourfold greater than the endogenous activity when synthesis was measured by ^{3}H-TMP incorporation and 23-fold greater when measured by ^{3}H-GMP incorporation. When poly(rC) oligo(dG) (the preferred synthetic RNA template for the polymerase of oncornavirions) was used, synthesis of DNA was not significantly enhanced over endogenous activity (Table III). The DNA polymerase of JIII particles, unlike polymerase of oncornavirions, does not use poly(rC) oligo(dG) under conditions where it does use exogenous DNA.

Figure 2 shows an electron micrograph of material taken from the 1.18–1.22 density region of a sucrose gradient in which a JIII tissue culture pellet had been brought to equilibrium. The material had been removed, treated with phosphotungstate and examined by electron microscopy. Only relatively uniform particles were observed, approximately 25 nm in diameter, electron luscent with a tendency to aggregate. Substructure could not be resolved.

TABLE I. OPTIMAL STIMULATION BY NONIDET OF DNA POLYMERASE ACTIVITY.[a]

Percent of Nonidet P-40 added[b]	Polymerase activity ^{3}H-TMP incorporation (cpm)
None	268
0.005	325
0.010	455
0.025	489
0.050	831
0.075	1176
0.100	1679
0.150	1092
0.200	891

[a] Twenty-five-microliter aliquots from the fraction in the 1.18–1.22 density region of the gradient with the highest enzyme activity were assayed for DNA polymerase using varying concentrations of Nonidet.

[b] Twenty-five microliters of Nonidet was preincubated with the enzyme preparation at 4° for 30 min prior to enzyme assay.

Presence of antigen(s) of extracellular JIII particles in cells derived from various species. Immune serum prepared against the particles and conjugated with fluorescein isothiocyanate-stained acetone-fixed JIII cells when tested with the direct fluorescent antibody method. The staining was generally distributed throughout the cytoplasm of the cell and was of a particulate nature (Fig. 3). The intensity of staining (1+-4+) and percentage of cells stained in each preparation varied from passage to passage, generally with a range of 10–100% of cells staining in any given preparation. Unfixed JIII cells were also stained by the conjugated JIII antiserum, indicating the presence of the antigen(s) on the surface of cells. The antiserum gave positive staining out to a 1:32 dilution.

The following experimental results determined that the staining reaction on JIII cells was specific: (a) conjugated preimmune serum did not stain the cells; (b) unconjugated immune serum gave a positive staining reaction with the indirect fluorescent antibody test using conjugated goat anti-rabbit gamma globulin, thus, indicating that globulins (antibodies) are responsible for the

TABLE II. EFFECT OF RNASE ON DNA POLYMERASE ACTIVITY OF PARTICLES FROM JIII CELL CULTURES.[a]

	DNA polymerase activity ^{3}H-TMP incorporation (cpm)					
	No Nonidet[b]			0.1% Nonidet		
Preparations	No RNase	RNase[b]	% Inhibition	No RNase	RNase	Inhibition (%)
1	215	188	12	746	535	28
2	815	1183	–	1120	826	26
3	448	374	16	1628	874	46
4	268	284	–	1679	1541	8

[a] Twenty-five-microliter aliquots from the fraction in the 1.18–1.22 g/cm^3 density region of the gradient with the highest enzyme activity were assayed for DNA polymerase activity with and without RNase treatment in the presence and absence of Nonidet treatment.

[b] Two-hundred micrograms of RNase and 0.1% Nonidet were preincubated with the enzyme preparation for 30 min at 4° prior to enzyme assay.

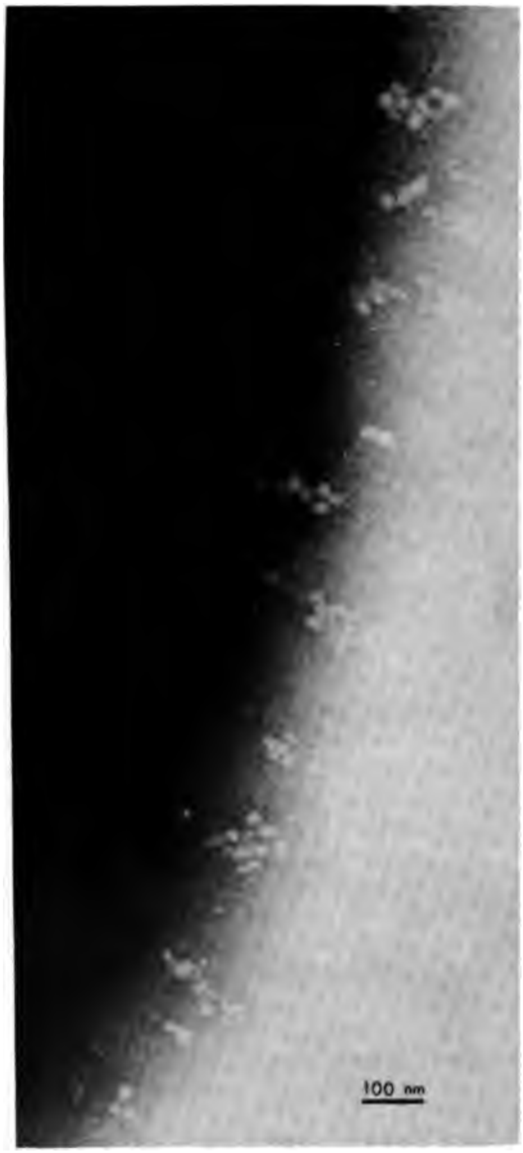


FIG. 2. Electron micrograph of particles from the 1.18-1.22 g/cm^3 density region of the gradient after staining with phosphotungstic acid.

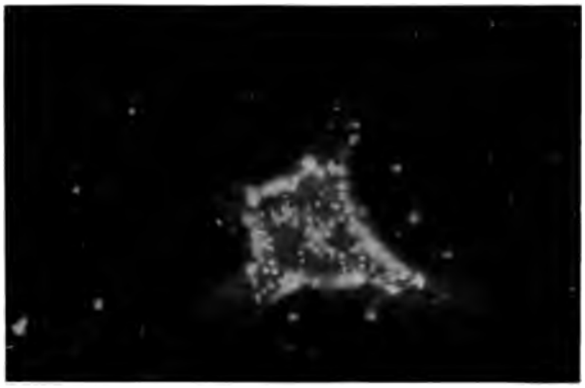
staining; (c) staining of the conjugated immune serum could be blocked completely by preincubation of JIII cells with unconjugated immune serum, but not with unconjugated preimmune serum or PBS; and (d) absorption of the conjugated immune serum with cell powder prepared from JIII cells removed the staining capacity of the conjugate, but absorption with cell powders prepared from nonmalignant human tonsilar lymphocytes had little or no effect. These results indicate that the staining observed is due to an antigen-antibody reaction in the immune serum opposed to nonspecific attachment of fluorescein dye, and that the detectable antigen is present on JIII cells but not on nonmalignant human lymphocytes.

Experiments were performed to determine if this antigen(s) on JIII cells was also present on other types of cells. Table IV gives a list of the types of cells tested with antiserum against JIII particles using the fluorescent antibody test. Only the Z-597 cell line, a continuous cell line derived from a patient with granulocytic leukemia, gave a positive staining reaction which was comparable to that observed on JIII cells. In another experiment, JIII antiserum stained both JIII and Z-597 cells at a 1:32 dilution using the direct fluorescent antibody test but specific staining characteristic of JIII cells was not observed with human fibroblasts and tonsilar lymphocytes.

Discussion. Very similar extracellular particles were detected in the culture media from two continuous cell lines (JIII and Z-597) derived from cases of human leukemia. JIII particles were related antigenically to the cells in both cultures, but not to eleven other continuous, primary, or uncultured cells derived from a wide range of species including chicken, mouse embryo, and human (Table IV). Further, human tonsilar lymphocytes failed to adsorb antibodies made against JIII particles.

While this suggests the particles may be related to the malignant aspect of the JIII cell, they do not relate to known oncornaviruses which also are released in culture from transformed cells. They resemble the oncornaviruses only in that they synthesize DNA using endogenous templates, primers, and polymerase, are stimulated by Nonidet, and are partially inhibited by RNase. They differ in density (1.18-1.22 vs 1.15-1.18), in size (25 nm vs 100 nm), in failing to use the exogenous template poly(rC) oligo(dG), and in containing some DNA as well as RNA.

The JIII particles more closely resemble certain particles of unknown significance



Acetone fixed JIII cells stained by a 1:8 dilution of antiserum prepared against JIII particles. ×400.

III. USE OF EXOGENOUS TEMPLATES BY THE DNA POLYMERASE OF JIII PARTICLES FROM SUCROSE GRADIENTS[a].

	^{3}H-TMP incorporation (cpm)[c] Fraction number			^{3}H-dGMP incorporation (cpm) Fraction number		
plate[b]	10	11	12	10	11	12
	147	177	164	59	85	112
thymus	489	750	286	804	2005	559
ligo(dG)	–	–	–	75	146	81

ty-five microliter aliquots from three factions (10, 11, 12), which had the highest enzyme activity in the density region of the gradient, were each assayed for DNA polymerase activity either with or without templates.

concentration of templates in the enzyme reaction mixture was 20 μg/ml.

zyme reactions were performed after preincubation with 0.1% Nonidet.

E IV. STAINING REACTIONS OF DIFFERENT CELLS WHEN TESTED WITH THE JIII ANTISERUM USING THE FLUORESCENT ANTIBODY TECHNIQUE.

Cell type	Type of culture	Dilutions of antisera	Fluorescent antibody test	Staining reaction
ukemia (JIII)	Continuous	1:32	DF	+
		1:64	IF	+
		1:8	DU	+
ukemia (Z-597)	Continuous	1:8	DF	+
nkey kidney (Vero)	Continuous	1:4	DF	–
		1:8	DF	–
onkey kidney (LLCMK$_2$)	Continuous	1:2	DF	–
rvical carcinoma (HeLa)	Continuous	1:4	DF	–
		1:8	DF	–
ney	Primary	1:2	DF	–
acrophages	Primary	1:2	DF	–
ster kidney (BHK21)	Continuous	1:2	DF	–
nsilar lymphocytes	Not cultured	1:8	DU	±
		1:8	DU	–
ng (WI 38)	Diploid	1:4	DF	–
		1:8	DF	–
dney (RK13)	Continuous	1:2	DF	–
broblasts	Diploid	1:4	DF	±
		1:8	DF	–
s mouse embryo	Primary	1:8	DF	–
		1:4	IF	–

F, direct fluorescent antibody method on fixed cells; IF, indirect fluorescent antibody method on fixed , direct fluorescent antibody method on unfixed cells.

previously detected in medium from a variety of cultured malignant cells and in certain human plasma. Particles with a density of 1.15-1.21 containing RNA and DNA (like JIII particles) were detected by Long *et al.* (6) in the medium of cultured spleen cells obtained from a patient with Hodgkin's Disease. Narayan and Rounds (7) detected 10-12 nm ring shaped particles in medium from cultured human adenocarcinoma cells. These also contained both RNA and DNA but had a density (1.6-1.19) similar to oncornaviruses. The plasma of two patients with chronic lymphocytic leukemia yielded to Kiessling *et al.* (8) particles with properties common to JIII particles. Both particles had similar densities and contained a DNA polymerase, which was stimulated by Nonidet and by exogenous DNA but not by synthetic RNA templates, poly(rC) oligo(dG) (JIII particles), and poly(rI-rC) (Kiessling's particles).

The release of complex particulates from cells in culture might be viewed as an aberration of some normal (albeit unknown) function of the cell or an additional example of a cytopathology induced by an unknown virus.

The development of a fluorescent antibody against one or more antigens of the particulate allows use of the antigen as a marker to learn if the particle can be replicated in those cells in which the antigen is presently absent. Information concerning the distribution of the antigen(s) in various pathologic human states might also be meaningful in establishing the significance of the particle. How fluorescent antibody might also facilitate collection of such relevant data will be illustrated in a separate communication.

Summary. Extracellular particles, with a density of 1.18-1.22 g/cm^3 in sucrose, were detected in the culture medium of a continuous cell line (JIII) derived from a patient with monocytic leukemia. These particles contained RNA, DNA, and a DNA polymerase. They synthesized DNA with endogenous templates and primers and also used exogenous DNA but not poly(rC) oligo(dG) as a template. Pretreatment with Nonidet P-40 stimulated DNA polymerase activity while treatment with ribonuclease partially inhibited the enzyme activity. Fluorescent antibodies made to the particles stained both JIII and Z-597 cells derived from human leukemias but not other types of human or nonhuman cultured cells tested. The particles do not appear to be oncornaviruses but may be a particulate antigen associated with malignant cells of hemopoietic and lymphoid origin.

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/arrows of an Antigen Released from Continuous Cell ived from Human Leukemia[1] (39460)

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cology, NIH, Bethesda, Md. D-17 cells, a canine cell line, uninfected and infected with simian sarcoma virus (SSV) were obtained from Dr. John Riggs. JIII cells, derived from a case of monocytic leukemia, were purchased from the American Type Culture Collection Cell Repository, Rockville, Md. All cells were grown in Eagle's minimal essential medium and Hank's balanced salt solution with 10% fetal calf serum and antibiotics. Cells were passaged when they reached confluency.

Antisera. Antiserum against disrupted RLV was obtained from Dr. Paul Levine, NIH, Bethesda, Md. and antiserum against disrupted SSV from Dr. T. G. Kawakami, University of California, Davis, Calif. Preparation of antiserum against JIII particles and conjugation with fluorescein isothiocyanate was described previously (8).

Bone marrow preparations. Two separate series of bone marrow films from patients with a variety of disorders were examined. The first series was obtained from the Collection of the Simpson Memorial Institute, University of Michigan and had been stored at −20° from 1-8 months. These films were fixed in acetone and tested with a 1:8 dilution of JIII antiserum using the direct fluorescent antibody method. A second series was tested to confirm the results of the first. These preparations were acetone-fixed the same day that the specimen was taken from the patient and tested with a 1:8 dilution of JIII antiserum within a week of that time. Only mononuclear leucocytes showing fluorescent intensity greater than 2+ (scale 1+ to 4+) in the cytoplasm and not in an area of dye trapping (at the edge of the slide) were considered positive. Coverslips containing no positive cells must have displayed 100 negative leucocytes to be evaluated as negative. All tests were performed at least

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twice on each sample and were read independently by two persons without knowledge of the patients diagnosis.

Results. Antigenic relationship of JIII particles to oncornaviruses. D-17 cells either uninfected or infected with SSV, and JLSV-9 cells uninfected or infected with RLV were grown on coverslips in Leighton tubes until they reached confluency. The cells were then fixed in acetone prior to testing. With the indirect fluorescent antibody method, a 1:10 dilution of bovine antiserum made against SSV and a 1:20 dilution of conjugated antibovine serum stained D-17 cells infected with SSV but not the uninfected cells. Unconjugated normal bovine serum did not stain these cells. A 1:10 dilution of monkey antiserum made against RLV and a 1:10 dilution of conjugated antimonkey serum stained JLSV-9 cells infected with RLV but not the uninfected cells. Normal monkey serum did not stain these cells. These results ensure the presence of viral antigens in the virus infected cells and not in the uninfected cells.

Undiluted JIII antiserum did not stain either the D-17 cells infected with SSV or JLSV-9 cells infected with RLV using the direct fluorescent antibody method. Neither a 1:4 or 1:8 dilution of unconjugated JIII antiserum with a 1:20 dilution of conjugated antirabbit serum stained either of these virus infected cells using the indirect fluorescent antibody method.

In another series of experiments, acetone-fixed JIII cells were tested with both JIII antiserum and antisera made against either RLV or SSV. A 1:8 dilution of conjugated JIII antiserum stained JIII cells with the direct method, but a 1:5 dilution of either SSV or RLV antisera did not stain these cells with the indirect method.

Presence of antigen of JIII particles on human bone marrow cells. The list of diseases of patients whose preparations were tested in the first series of tests is given in Table I. All preparations were tested with a 1:8 dilution of JIII antiserum using the direct fluorescent antibody method. Certain preparations from patients with Hodgkin's disease, chronic lymphocytic leukemia, chronic granulocytic leukemia, anemia, and abnormal white blood cells were stained by the JIII antiserum. Table II gives the percentage of positive bone marrow preparations according to the general type of disorder. Preparations from patients with lymphoma were stained more frequently (45% positive) than those from patients with leukemia (11%). Bone marrow films from patients with nonmalignant blood disorders were positive with a frequency (25%) between the two.

The list of diseases of patients whose preparations were tested in the second series is listed in Table I. Again certain preparations from patients with Hodgkin's disease were positive as well as preparations from patients with multiple myeloma, atypical lymphocytes, red blood cell aplasia, renal failure, and one patient with undetermined diagnosis. None of the preparations from patients with leukemia stained. Examination of these results grouped according to the general disorder (Table II) revealed results similar to those seen in the first series of tests, except the percentage of positive preparations was slightly lower in the second series. Table II also gives the percentage of positive results of the first and second series of tests combined.

Characteristics and specificity of staining on bone marrow cells. Generally, lymphocytes and monocytes were the types of bone marrow cells which gave positive staining. The percentage of positive cells (only mononuclear cells considered) ranged from 1 to 50%. Figure 1 is a photomicrograph of a positive leucocyte which demonstrates uniform cytoplasmic fluorescence. Conjugated preimmune rabbit serum did not stain the bone marrow cells nor did conjugated antiserum prepared against antigens unrelated to JIII particles. Absorption of JIII antiserum with JIII cell powder reduced the dilution of antiserum which could stain the bone marrow cells from 1:8 to 1:2. Absorption of antiserum with tonsilar lymphocyte powder had minimal effect on the staining capacity of the antiserum when tested on bone marrow cells. This result suggests that the antigen being detected on the bone marrow cells is related or identical to the antigen of JIII particles.

Statistical analysis. There was not a significant difference between the first and sec-

TABLE I. FLUORESCENT ANTIBODY TESTS OF BONE MARROW FILMS FROM PATIENTS WITH VARIOUS DISEASES WITH JIII ANTISERUM[a].

First Series		Second Series	
Disease	No. positive/ total	Disease	No. positive/ total
Acute myelomonocytic leukemia	0/12	Hodgkin's Disease	4/11
Chronic lymphocytic leukemia	2/9	Non Hodgkin's lymphomas	4/15
Chronic granulocytic leukemia	1/4	Reticulum sarcoma	1/2
Acute lymphocytic leukemia	0/1	Histiocytic lymphoma	1/1
Histiomonocytic leukemia	0/1	Poorly differentiated lymphocytic lymphoma	1/5
Hodgkin's Disease	3/7	Well differentiated lymphocytic lymphoma	0/12
Anemia	2/9	Unclassified lymphoma	1/15
Lymphadenapathy	0/2	Chronic granulocytic leukemia	0/2
Abnormal white blood cells	1/1	Prolymphocytic leukemia	0/1
Lymphomas	2/4	Acute lymphocytic leukemia	0/1
Scleroderma	0/2	Acute myelomonocytic leukemia	0/12
		Chronic lymphocytic leukemia	0/3
		Erythroleukemia	0/1
		Cancer metastasis	0/3
		Multiple myeloma	1/3
		Atypical lymphocytes	1/1
		Agranulocytosis	0/1
		Polycythemia	0/1
		Red cell aplasia	1/1
		Anemia	0/13
		Cirrhosis	0/1
		T. B. abscess	0/1
		Renal failure	1/2
		Kidney transplant	0/1
		Seizures syndrome	0/1
		Arthritis	0/1
		Sarcoidosis	0/1
		Undetermined diagnosis	1/1

[a] The direct fluorescent antibody test was used with a 1:8 dilution of JIII antiserum.

TABLE II. RESULTS OF FLUORESCENT ANTIBODY TEST ON BONE MARROW FILMS GROUPED ACCORDING TO THE GENERAL DISORDER.

	First series		Second series		First and Second series	
Disease	No. positive/total	Positive (%)	No. positive/total	Positive (%)	No. positive/ total	Positive (%)
All lymphomas	5/11	45	9/26	34	14/37	38
All leukemias	3/27	11	0/20	0	3/47	6
Other cancers	–	–	1/6	14	1/6	16
Nonmalignant blood disorders	3/12	25	2/7	28	5/19	26
Nonmalignant disorders	0/2	0	1/8	12	1/10	10

ond series of tests when the fraction of positive or negative preparations from lymphoma patients were compared by chi square tests ($\chi^2 = 0.38$, p 70.50). There were not enough positive preparations from patients with leukemia to do chi square tests to compare series 1 and 2, but those results and results of preparations from patients with nonmalignant blood disorders were very similar. (Table II). Using the combined groups, chi square tests were also used to determine whether the fraction of positives in preparations from patients with leukemia and those with lymphoma are significantly different. A chi square value of 13.42 ($p <$ 0.001) was calculated. A chi square value of 1.00 ($p > 0.30$) was calculated when results from tests of preparations from patients

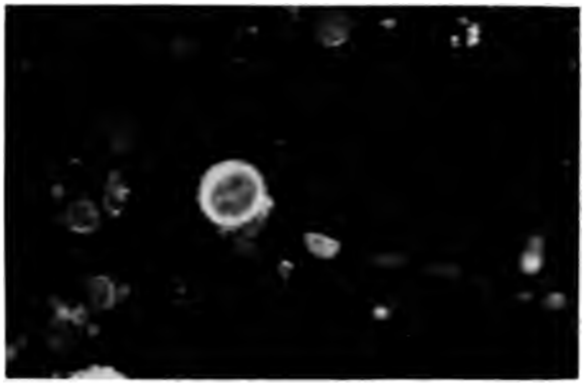

FIG. 1. Bone marrow leucocyte with cytoplasmic fluorescence. This preparation was stained with a 1:8 dilution of the JIII antiserum using the direct fluorescent antibody test. 400 ×.

with lymphoma and nonmalignant blood disorders were compared. There appears to be a significant difference in results to the test of preparations from patients with leukemia and lymphoma but not of results of preparations from patients with lymphoma and this selection of nonmalignant blood disorders at a 5% level.

Another series of statistical tests were performed which dealt with comparing five different characteristics of the patients with lymphoma to the fluorescent antibody test results. Chi square tests were done analyzing the variables sex and stage of disease, the Fisher exact test was done on the variables, treatment of patients and bone marrow involvement with malignant cells, and the t test was done on the variable, age of patients at time of diagnosis. There was a significant relationship between age of the patients and the fluorescent antibody test result $t = 2.82$ ($p < 0.01$).

Positive and negative fluorescent antibody results were grouped into age deciles in order to further examine the relationship between age of patients and the fluorescent antibody results. Table III shows the number of positive and negative preparations in each age decile. The difference was small between the number of positive and negative preparations in the age deciles up to age 50, since 23 preparations were examined, and 12 were positive while 11 were negative. In contrast, the results of preparations from patients over the age of 50 showed that of 14 tested, only 2 were positive and 12 were negative. It appears that patients with lymphomas over age 50 are less likely to have the antigen of JIII particles on their cells as those under 50.

TABLE III. AGE DISTRIBUTION (AGE AT TIME OF DIAGNOSIS) OF PATIENTS AND FLUORESCENT ANTIBODY TEST RESULTS.

	Numbers of patients		
Age decile	Positive	Negative	Total
11-20	3	0	3
21-30	2	3	5
31-40	2	5	7
41-50	5	3	8
51-60	2	8	10
61-70	0	4	4

Discussion. The antigen displayed on bone marrow cells with fluorescent antibody is similar or identical with the JIII particulate antigen(s) since the antibody is adsorbed by JIII cell powder but not by tonsilar lymphocyte powder. It is unlikely the antigen is a histocompatibility antigen because of the age distribution of its frequency. It is clearly unrelated to representative oncornaviruses. The JIII antigen is present only in the bone marrow of some leukemia and lymphoma patients. It is not known whether the bone marrow samples which did react positively to the JIII antibody would have reacted positively with antibodies prepared to plasma particles or to oncornaviruses. These malignancies may have multiple etiologies. The bimodal age distribution of Hodgkin's disease suggests this, as well as a different pathology, course and prognosis in younger patients compared to those over 50 years of age (9). If the JIII antigen is etiologically significant, this may account for the high frequency of the JIII antigen in younger people with lymphoma. From animal models of infectious etiologic agents, acute productive infections of cells are to be anticipated in addition to malignant transformation, ie., the presence of the agent is likely not restricted to tumor cells. This may be the meaning of the positive preparations from patients with nonmalignant blood disorders.

The significance of the JIII antigen might be clarified by determining its presence in pathologic specimens, in conjunction with simultaneous tests for other etiologic candidates and from a study of lymphomas arising in epidemiologic clusters.

Summary. Fluorescent antibodies prepared against extracellular particles from a continuous culture of cells derived from a monocytic leukemia stained JIII cells but not cells infected with Rauscher leukemia virus or simian sarcoma virus. These antibodies reacted with 38% of bone marrow preparations from patients with lymphoma, 26% of preparations from patients with nonmalignant blood disorders and 6% of preparations from patients with leukemia. Bone marrow films from patients with lymphoma over the age of 50 stained less frequently than those from patients under 50. These particles released from JIII cells are not antigenically related to two of the commonly studied oncornaviruses, but may be indicative of the etiology or disease process of lymphoma in young patients.

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Effect of Tryptophan and its Metabolites on Gastric Emptying of Liquid Meals in Dogs (39461)

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In a previous study (1) we found that tryptophan slowed gastric emptying significantly (60% inhibition) in concentrations about 10× lower than 25 other amino acids including phenylalanine. We concluded that there appeared to be a specific tryptophan receptor which was probably different from the classical osmoreceptor (1-3).

Tryptophan is well absorbed by the small intestine and is largely metabolized in the liver (4). It is possible, therefore, that tryptophan or its metabolites may act, after absorption, directly on the stomach to slow emptying.

The aim of this study was to determine whether tryptophan or some of its metabolites act directly on the stomach or brain rather than via gastrointestinal receptors. This was done by instilling tryptophan metabolites into the gut or infusing tryptophan and some metabolites intravenously (Fig. 1).

Methods and materials. Studies over a period of several months were made in five healthy dogs weighing 14 to 19 kg. Each dog was equipped with a Thomas (5) cannula to form a gastric fistula. The dogs were fasted 18 hr before testing but were allowed water *ad lib.*

Procedures. The dogs were brought to the laboratory and their stomachs were rinsed with tap water via the gastric fistula to clear accumulated secretions. This rinse was allowed to drain by gravity. A plastic adapter with a stop-cock was then connected to the gastric cannula through which the test meals were instilled and drained. Two 25-min test meals were given in succession with 35 min between each meal. The first meal (300 ml at 37°) was a control and consisted of deionized water with phenol red (40 mg liter^{-1}), a nonabsorbable marker dye and was instilled via the gastric fistula into the stomach in less than 45 sec. After 25 min, the contents of the stomach were drained completely by gravity through the gastric fistula. Immediately after the gastric contents were collected, 200 ml of a "wash" solution of deionized water was instilled into the stomach to recover any residual contents and then drained. The gastric fistula was left open and gastric secretions were allowed to drain by gravity in the 35 min interval between tests. The second meal containing the test substance and phenol red was administered and collected in precisely the same fashion as the first.

These techniques were varied slightly in the intravenous studies. For each experiment, a polyethylene catheter (PE 50, Intramedic, Clay Adams Inc., N.Y.) was inserted into a dog's hind leg vein. A syringe pump (Harvard Apparatus, Dover, Mass.) was used to infuse normal saline or the test substance into the vein at 25 ml hr^{-1}. As the control, the dog was infused for 1 hr with normal saline and during the last 25 min a deionized water meal was given. The test substance was then added to the iv infusion and the mixture infused for 1 hr, the last 25 min of which a deionized water meal was given. The collection of the test meals was identical to that described above.

The volumes of the residual gastric contents and wash solutions were measured to the nearest 1 ml and samples were saved for determination of phenol red concentration. Phenol red concentration after alkalinization was measured spectrophotometrically at a wavelength of 558 nm.

Test Meals and Substances. All meals were 300 ml in volume with phenol red (40 mg liter^{-1}). The osmolalities were the same as the individual concentrations. In the first series of studies the test meals contained DL-kynurenine (5 m*M*), 5-hydroxytryptophan (5 m*M*), or 5-hydroxytryptamine (serotonin) in a concentration of 20 m*M* (20

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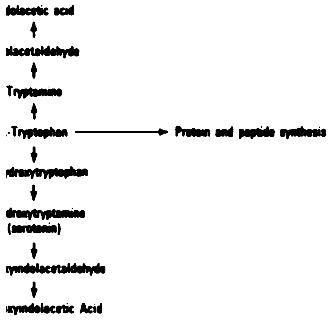

he major metabolic pathways of tryptophan.

TABLE I. THE EFFECT OF INSTILLED TRYPTOPHAN METABOLITES ON GASTRIC EMPTYING.

Metabolite	Number of dogs	Mean control (ml ± SE)	Mean Test (ml ± SE)	P
5-Hydroxytryptamine (20 m*M*)	4	33 ±6	44 ±19	>0.05
DL-Kynurenine (5 m*M*)	4	25 ±5	42 ±12	>0.05
5-Hydroxytryptophan (5 m*M*)	2	23 ±3	53 ±18	–

droxytryptophan (5 m*M*) was studied in two dogs only because of toxicity (extreme restlessness).

Effect of intravenous tryptophan and metabolites. All concentrations of L-tryptophan, 3-indolepyruvic acid, 5-hydroxytryptamine and DL-kynurenine failed to cause significant slowing of gastric emptying ($p > 0.05$) (Table II).

Discussion. Previous studies have indicated that tryptophan is a powerful inhibitor of gastric emptying and is unique among amino acids for this action (1). Those studies, however, did not determine whether the effect of tryptophan was due to tryptophan itself or some of its metabolites.

In the present study we did not find an effect on gastric emptying with large doses of the tryptophan metabolites 5-hydroxytryptamine, DL-kynurenine, and 5-hydroxytryptophan following instillation into the gut (Table I). Furthermore, when these same metabolites as well as 3-indolepyruvic acid and L-tryptophan itself were infused intravenously, no statistically significant slowing of emptying occurred (Table II). These studies

TABLE II. The Effect of Intravenous Tryptophan and Metabolites on Gastric Emptying.

	Δ[a] mean volume (ml ± SE) at various concentrations				
Substance	2 m*M*	5 m*M*	10 m*M*	20 m*M*	50 m*M*
L-Tryptophan	–	– 6 ± 17	–	9 ± 19	–13 ± 12
3-Indolepyruvic	–19 ± 8	29 ± 30	13 ± 20	–	–
5-Hydroxytryptamine	65 ± 43	–	65 ± 30	–	–
DL-Kynurenine	20 ± 33	62 ± 23	48 ± 23	–	–

[a] The Δ volume is the difference between control and following iv infusion. Negative values indicate that control was greater than the test situation.

indicate that tryptophan is the agent which delays gastric emptying and not its metabolites and that it works by exciting receptors in the gut and not by direct action on the stomach or brain.

Summary. The effects of tryptophan and its metabolites were studied to determine whether the known inhibiting effect of tryptophan on emptying was due to local or systemic effects or due to tryptophan metabolites. In five dogs with chronic gastric fistulas, instillation of 300 ml of DL-kynurenine (5 m*M*), 5-hydroxytryptophan (5 m*M*), or 5-hydroxytryptamine (20 m*M*) into the gut did not slow gastric emptying. Furthermore, iv infusion of L-tryptophan (5, 20, 50 m*M*), DL-kynurenine (2, 5, 10 m*M*), 5-hydroxytryptamine (2 m*M*, 10 m*M*), and 3-indolepyruvic acid (2, 5, 10 m*M*) also did not slow gastric emptying.

These studies indicate that tryptophan slows gastric emptying by exciting a receptor in the gut and not by a direct effect on the stomach or brain or via its major metabolites.

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bution of Luminal Concentration of Nutrients and Osmolality to Postprandial Intestinal Hyperemia in Dogs[1] (39462)

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e anticipation and ingestion of food generalized cardiovascular response vating the sympathetic nervous sys- e cardiovascular adjustments during tprandial period are limited to a hy- a in the digestive organs (1–3). The mia begins within 5 to 15 minutes ng oral ingestion of food. Recent further indicate that the hyperemia astrointestinal tract is confined to the l layer of the segment containing (4–6). These studies suggest that the mia may be related to absorptive es and/or interaction of chyme with cosa. The present study was designed ss if the concentration of nutrients osmolality of chyme in the gut lumen nificant factors determining the de- postprandial intestinal hyperemia.

ods. Fasted mongrel dogs were anes- d with pentobarbital sodium (30 mg/ ntilated with a Harvard respirator, en heparin sodium (6 mg/kg) as an gulant. A loop of jejunum was exter- and divided into two segments, each l by a single vein (4). The veins were ted and the venous outflows were d into a reservoir from which the vas pumped back to the animal via a l vein. A rubber tube was placed into en of each segment for the introduc- d withdrawal of fluids. At all other he tube was connected to a Statham e transducer (Model No. P23Gb) to r intraluminal pressure. Both ends of gment were tied and the mesentery to exclude collateral flow. Systemic pressure was continuously moni- rough a femoral arterial cannula. mercially available dog food (Alpo, Allen Products Co., Allentown, Pa.; fat, 7.0%; protein, 12.0%; fiber, 1.5%; moisture, 78.0%; ash, 3.0%) was homogenized and its pH was adjusted to approximately 7.0 with $NaHCO_3$. To each can of this homogenate was added 750 mg of a pancreatic enzyme preparation[2] (Viokase, Viobin Corp., Monticello, Ill.) and the whole mixture was gently stirred with a magnetic stirrer for 5 hr to permit digestion. From this digested food, four mixtures of different concentrations were prepared. One was undiluted (100%); a second was diluted with distilled water to give a mixture which was 33% food in water, i.e., 1 part food, 2 parts water. The third was diluted to give a 20% and the fourth was 10% food in water. Aliquots of each of the four food mixtures were centrifuged (19,250*g*) and the pH and osmolalities of their supernatants determined (Beckman Expandomatic pH meter, Beckman Instruments, Incorporated, Advanced Osmometer, Advanced Instruments, Incorporated). The pH of 100, 33, 20, and 10% food mixtures were 6.40 ± 0.02, 6.53 ± 0.02, 6.57 ± 0.02, and 6.58 ± 0.04, respectively, and the osmolalities were 998.6 ± 30.3, 291.3 ± 5.3, 183.0 ± 5.8, and 94.0 ± 2.7 mOsm/kg (mean ± SEM). Seven and 12 cans of the dog food were treated as described above for the determination of pH and osmolality, respectively.

The protocol for all experiments consisted of three 15-min periods: Precontrol, test, and postcontrol. In the pre- and postcontrol periods, both segments contained 10 ml of normal saline. The luminal contents in the test period varied with each experiment. During the test period of the first series of experiments ($N = 7$), one segment contained 10 ml of one of the four food mix-

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investigation was supported by Research L 15231 from the National Institutes of This paper was abstracted in part in Physiolo- 68, 1974.

[2] The pancreatic enzyme preparation did not alter jejunal blood flow when placed into the lumen (unpublished observation).

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erved.

tures and the other segment 10 ml of normal saline so that the effects of each of these mixtures could be studied using saline as a control in the other segment. In the second series of experiments (*N* = 6), the hyperemic effects of 100, 33, and 20% food mixtures were compared. During the test period, one segment contained 10 ml of one of these three mixtures and the other segment 10 ml of one of the other two mixtures. Since 100, 20, and 10% food mixtures were not isotonic to plasma, the effects of these mixtures might be due, in part, to their osmolalities. The third series of experiments (*N* = 6) was designed to test this possibility. In order to eliminate the hypotonic effect of 20 and 10% food mixtures, isotonic mixtures of these foods (320.0 ± 12.3 and 281.3 ± 6.0 mOsm/kg, respectively) were made from a portion of these mixtures by addition of appropriate amounts of NaCl. During the test period, one segment contained 10 ml of a hypotonic mixture while the other segment contained 10 ml of the isotonic mixture of the same concentration. The hypertonic 100% food could not be made isotonic without decreasing its concentration. Therefore, a 30% solution of a nonabsorbable substance, polyethylene glycol (PEG), having the same osmolality (1000 mOsm/kg) as that of 100% food was made. During a test period, one segment contained 10 ml of 100% food while the other segment contained 10 ml of 30% PEG. In the three series, all solutions introduced into the intestinal lumen were at 37°.

Control or test mixtures remained in the lumen for 15 min during which venous outflows from both segments were simultaneously collected in graduated cylinders in 3-min samples with a 1-min interval between collections. Aliquots of venous blood of the last collection were retained for determination of osmolality (Advanced Instruments Osmometer). After each period, the lumen contents were withdrawn, their volumes measured, and the lumen was gently washed with warm normal saline.

Results. Systemic arterial pressure which ranged from 116 to 165 mm Hg (136.8 ± 2.7 mm Hg, mean ± SEM) in all three series of experiments was not significantly altered by luminal placement of any food mixture, saline, or PEG. After luminal placement of 100, 33, or 20% food mixture, venous outflow gradually increased, reached a plateau by 8 to 11 min, and remained at that level for the next 4 min. The venous outflow measured 8 to 11 min following the placement of any food mixture was statistically the same as that measured during the last 3-min period. Therefore, only the values from the last 3-min flow collection were used for expressing and analyzing the data.

Series I (Table I). Luminal placement of 100, 33, or 20% digested food mixture significantly increased venous outflow, but 10% food did not. No significant change in flow occurred in the control segment into which normal saline was placed successively in the three periods. The lumen volume significantly increased (+2.5 ± 0.8 ml) with 100% food in the lumen, did not change with saline, 33 or 20% food, and decreased with 10% food (−3.5 ± 0.8 ml). Only 100% food significantly increased venous osmolality (+10.6 ± 2.4 mOsm/kg from a control of 293.3 ± 1.6 mOsm/kg).

Series II (Table II). The hyperemic effect of luminal placement of 100% food was significantly greater than that of 33 or 20% food (comparisons 1 and 2). The hyperemic effect of 33 and 20% food mixtures was similar. Again, only 100% food in the lumen significantly increased lumen volume and venous osmolality.

Series III (Table III). While 100% food markedly increased flow, 30% PEG did not significantly alter flow. Both solutions, however, equally increased venous osmolality and lumen volume. Both isotonic and hypotonic 20% food mixtures increased flow to the same extent and did not significantly alter venous osmolality or lumen volume. Neither isotonic nor hypotonic 10% food significantly altered flow, but hypotonic 10% food significantly decreased lumen volume.

Discussion. The aim of this study was to assess if the concentration of nutrients and the osmolality of chyme in the intestinal lumen are significant factors determining the degree of intestinal hyperemia during

TABLE I. MEAN ± SEM VENOUS OUTFLOW (ML/MIN/100G) FROM TWO ADJACENT JEJUNAL SEGMENTS[a].

	Experimental segment			Control segment		
Lumen content	Saline	Food	Saline	Saline	Saline	Saline
100% food	47.4 ± 5.7	65.6 ± 6.6[b]	47.7 ± 6.6[b]	55.1 ± 8.4	51.8 ± 8.5	59.1 ± 11.7
33% food	57.0 ± 9.8	66.5 ± 10.4[b]	52.9 ± 8.1[b]	51.7 ± 7.7	47.1 ± 6.7	46.3 ± 5.8
20% food	49.4 ± 7.9	60.5 ± 8.3[b]	50.5 ± 6.6[b]	51.4 ± 6.9	50.8 ± 5.8	46.1 ± 5.7
10% food	55.3 ± 7.6	59.0 ± 8.6	54.4 ± 9.5	59.2 ± 12.4	54.3 ± 11.7	52.6 ± 11.8

[a] $N = 7$ for each food mixture.

[b] Value significantly different from preceeding value, $p < 0.05$ (paired Student's t test).

TABLE II. COMPARISON OF THE EFFECTS OF LUMINAL PLACEMENT OF 100, 33, AND 20% FOOD ON VENOUS OUTFLOW, VENOUS OSMOLALITY, AND LUMEN VOLUME[a]

	Segment A	Segment B	D = A − B[b]
Comparison 1	100% food	33% food	
Blood Flow (%)	+26.9 ± 4.4[c]	+10.4 ± 1.3[c]	+16.5 ± 5.9[c]
Osmolality (mOsm/kg)	+8.3 ± 2.3[c]	+1.5 ± 1.3	+6.8 ± 2.1[c]
Lumen volume (ml)	+1.9 ± 0.1[c]	+0.2 ± 0.4	+1.7 ± 0.4[c]
Comparison 2	100% food	20% food	
Blood flow (%)	+24.4 ± 6.2[c]	+10.3 ± 2.9[c]	+14.1 ± 4.5[c]
Osmolality (mOsm/kg)	+10.5 ± 2.5[c]	+0.8 ± 1.2	+9.7 ± 3.4[c]
Lumen volume (ml)	+2.1 ± 0.7[c]	−0.5 ± 0.4	+2.6 ± 0.9[c]
Comparison 3	33% food	20% food	
Blood flow (%)	+18.3 ± 4.0[c]	+17.3 ± 4.7[c]	+1.0 ± 2.4
Osmolality (mOsm/kg)	+2.0 ± 1.3	+0.0 ± 0.6	+2.0 ± 1.2
Lumen volume (ml)	+0.4 ± 0.3	−0.6 ± 0.7	+1.0 ± 0.5

[a] N = 6 for each comparison. The values are changes from precontrol values. Comparisons 1 and 2 were performed in the same animals with food from the same cans, but comparison 3 was performed in another group of animals. Data from the latter group are also presented in Table III.

[b] D = the difference in changes produced by two paired food mixtures.

[c] Values are statistically significant, $p < 0.05$ (paired Student's t test).

digestion. As shown in Table I, luminal placement of 100, 33, or 20% food mixture significantly increased venous outflow, in contrast, placement of a 10% food mixture did not significantly alter flow. These data suggest that the concentration of nutrients in the lumen must exceed a certain value to produce local hyperemia. The hyperemic effect of 100% food was significantly greater than that of 33 or 20% food (comparisons 1 and 2, Table II) indicating that the greater the concentration of nutrients in the chyme the greater is the resultant hyperemia. However, a slight difference in the concentration does not appear to produce an appreciable difference in the hyperemic effect because 33 and 22% food mixtures increased flow to the same extent (comparison 3, Table II).

Since the osmolalities of the four food mixtures are different, the differences in their vascular effects may be due to the differences in their osmolalities. However, as shown in Table III, a 30% PEG solution having the same osmolality as 100% food does not significantly alter venous outflow even though its effects on venous osmolality and lumen volume are qualitatively and quantitatively similar to those of 100% food. The hyperemic effect of 100% food therefore is not due to its hypertonicity. Also, neither the hyperemic effect of 20% food nor the lack of vascular effect of 10% food is due to their hypotonicity. As shown in Table III, keeping the concentrations of nutrients in the mixtures unchanged but raising their osmolalities to near isotonicity does not alter the vascular effects of the hypotonic 20 or 10% food mixtures. These data thus indicate that lumen osmolality, within a range of 180 to 1000 mOsm/kg is not a significant factor contributing to the local intestinal hyperemia produced by the presence of chyme in the jejunal lumen. We have also shown previously that changes in

TABLE III. Comparison of the Effects of Luminal Placement of Hypertonic PEG and 100% Food, Iso- and Hypotonic 20% Food, and Iso- and Hypotonic 10% Food on the Venous Outflow, Venous Osmolality, and Lumen Volume[a].

	Segment A	Segment B	D = A − B[b]
Comparison 1	100% food	30% PEG	
Blood flow (%)	$+37.4 \pm 7.9^c$	$+1.4 \pm 2.2$	$+36.0 \pm 6.5^c$
Osmolality (mOsm/kg)	$+13.2 \pm 3.9^c$	$+13.7 \pm 5.0^c$	-0.5 ± 5.1
Lumen volume (ml)	$+3.3 \pm 0.7^c$	$+4.2 \pm 0.6^c$	-0.9 ± 0.7
Comparison 2	I-20% food	H-20% food	
Blood flow (%)	$+18.7 \pm 6.6^c$	$+18.9 \pm 6.8^c$	-0.2 ± 3.5
Osmolality (mOsm/kg)	$+4.3 \pm 1.9$	$+0.5 \pm 2.0$	$+3.8 \pm 3.7$
Lumen volume (ml)	-0.4 ± 0.5	-0.9 ± 0.8	$+0.5 \pm 0.7$
Comparison 3	I-10% food	H-10% food	
Blood flow (%)	$+3.1 \pm 7.9$	$+7.6 \pm 5.4$	-4.5 ± 5.0
Osmolality (mOsm/kg)	$+2.3 \pm 2.9$	-5.3 ± 2.9	$+7.6 \pm 4.9$
Lumen volume (ml)	-1.3 ± 7.4	-4.3 ± 1.0^c	$+3.0 \pm 0.7^c$

[a] $N = 6$ for each comparison. The values are changes from precontrol values. I-20% and I-10% = Isotonic 20% and 10%. H-20% and H-10% = Hypotonic 20% and 10%.

[b] D = the difference in changes produced by two paired food mixtures.

[c] Values are statistically significant, $p < 0.05$ (paired Student's *t* test).

lumen pH, over a range of 2.5 to 11.0, do not significantly alter local blood flow (7, 8).

The finding that the hyperemia requires a presence of a certain amount of digested food in the lumen and is directly related to the concentration of digested food in the chyme suggests that some chemicals in the food mixture are vasoactive when placed into the lumen. We have found recently that the pancreatic enzyme preparation used in this present study is not vasoactive when placed into the lumen. We have also found that the vasoactive substances are suspended in the supernatant of the digested food since luminal placement of the supernatant increases blood flow, but placement of the precipitate or undigested food do not (unpublished observation). The vasoactive chemicals therefore seem to be digestive products of carbohydrates, lipids, and/or proteins. We have shown that glucose increases intestinal blood flow when placed into the lumen and the greater the concentration the greater is the hyperemia (4).

Summary. Intestinal blood flow is increased during digestion. This study assesses if the concentration of nutrients and/or osmolality of chyme in the intestinal lumen are factors determining the hyperemia. Six digested food mixtures containing different concentrations of nutrients and/or having different osmolalities were placed into the jejunal lumen, and their effects on local venous outflow compared. The 100% (999 mOsm/kg), 33% (291 mOsm/kg), and 20% (183 mOsm/kg) food mixtures all increased flow, but the 10% food mixture (94 mOsm/kg) did not. The hyperemic effect of 33 and 20% food was similar, but 100% food produced a greater increase in flow than did 33 or 20% food. Luminal placement of a 30% solution of a nonabsorbable substance polyethylene glycol (1000 mOsm/kg) did not alter flow. Also, the vascular effects of 20 or 10% food mixtures were not altered when these mixtures were made isotonic by the addition of NaCl. These studies indicate that lumen osmolality, within a range of 180 to 1000 mOsm/kg, is not a significant factor contributing to the local hyperemia occurring when nutrients are in the gut lumen. However, the concentration of nutrients in the lumen is a factor determining the local hyperemia.

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Human Lymphocyte Subpopulations: The Effect of Pregnancy[1] (39463)

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The pregnancy state has always been an immunological enigma in that the fetus can survive in the uterus across a histocompatibility barrier without undergoing transplantation rejection. That this may be related to a modification of immunological status of the mother is suggested by the observations that (i) her lymphocytes respond poorly to mitogenic stimulation, especially in the presence of autologous serum (1), (ii) skin graft survival is prolonged (2), and (iii) the response to tuberculin skin testing is depressed (3). The present investigations were carried out to ascertain if these phenomena might be due to changes in the pool sizes of the various lymphocyte subpopulations.

Methods. Subjects. Blood was drawn from two groups of women after obtaining their informed consent: The first group consisted of females in the third trimester of a normal pregnancy who were attending the UCLA prenatal clinic; the second group consisted of normal nonpregnant female volunteers of child-bearing age who had never taken oral contraceptives or other hormonal supplements or had not taken either within the previous 30 or more days.

Lymphocyte Preparations. Blood was drawn in heparinized tubes between 9 AM and 11 AM in all subjects. Total lymphocyte counts were determined by routine methods from total white blood cell counts and differential blood smears. Lymphocytes were separated from peripheral blood by Ficol-Hypaque sedimentation and then incubated with latex beads in serum-free media at 37° for 30 min to facilitate the later identification of monocytes, as previously described (4).

B-Lymphocytes. Two receptors found primarily on B-lymphocytes were identified, namely, the receptor for aggregated IgG (AggIgG) and the receptor for complement. The receptor for AggIgG was identified as described elsewhere (4). After being incubated with heat-aggregated gamma globulin, lymphocytes were washed and incubated with fluorescein-conjugated goat antihuman gamma globulin antisera. After being washed the cells were examined live under phase contrast and epifluorescent microscopy. Since the fluorescein-conjugated antisera had polyvalent activity, the assay did, in fact, identify lymphocytes with surface immunoglobulin and/or receptors for aggregated IgG.

The method for the detection of the receptor for complement was modified slightly from that of Mendes (5). Zymosan granules were disaggregated by sonication and washed in Hank's balanced salt solution (HBSS). They were sensitized with a 1:5 dilution of fresh or deep frozen human serum in HBSS. After washing and resuspending the complement-coated granules (ZyC) to 10^8/ml., 0.1-ml lymphocyte suspension (5×10^6/ml) was mixed with 0.1 ml ZyC and spun at 50*g* for 5 min. After overnight incubation at 4° the cells were resuspended, several drops of 0.1% crystal violet solution (to identify lymphocytes) were added, and a minimum of 400 cells were examined. A lymphocyte binding two or more granules was considered to have receptors for complement.

T-lymphocytes. T-lymphocytes were identified by their ability to bind sheep red blood cells (SRBC) as described elsewhere (4). Briefly 0.1 cc of a 0.5% SRBC solution supplemented with 20% fetal calf serum (previously absorbed with SRBC) was mixed with 0.1 cc of lymphocyte suspension (5×10^6/ml). After incubation at 37° for 5 min and spinning at 50*g* for 5 min, the mixture was incubated overnight at 4°. Just prior to examination, crystal violet was added and the cells were resuspended on a

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:otator for 2.5 minutes. Lympho-
ich bound three or more SRBC
sidered as T-lymphocytes.

:al Analyses. All results were cal-
.nd expressed as mean ± standard
'omparison between groups was
ng Student's *t* test.

'. The recoveries of mononuclear
n the interface of the Ficol–Hy-
:adient separation of blood were
ı pregnant and nonpregnant sub-
.2 ± 4.8% vs 64.2 ± 6.2%).

le I are shown the percentages and
numbers of lymphocytes and lym-
subpopulations in pregnant and
ıant females. Of particular note is
'egnant females there was a signifi-
ıction in cells with receptors for
as compared to their nonpregnant
arts ($p < 0.001$). Conversely, cells
ptors for complement (the other B
:er) were equal in numbers in both
The percentage of T-lymphocytes
:ased significantly in the pregnant
hough the absolute numbers were

sion. In many respects pregnancy is
ıt to an organ transplantation
ıistocompatibility barrier. The fact
fetus is seldom rejected (aborted)
in part at least, a reflection of a
:ion of the mother's immunological

mechanisms (6). By identifying and quantitating B- and T-lymphocyte subpopulations, which are thought to regulate humoral and cell-mediated immunity, the present investigation examined the hypothesis that such modification may result from changes in the absolute pool sizes of certain lymphocyte subpopulations. While the absolute numbers of cells with receptors for sheep red blood cells (SRBC) and for complement were similar in both pregnant and nonpregnant females, the absolute numbers of lymphocytes with receptors for aggregated IgG (AggIgG) were significantly reduced in pregnant females. In normal subjects these two lymphocyte subpopulations represent two discrete but partially overlapping subpopulations of the B-series. The present findings suggest that these two lymphocyte subpopulations are affected to different degrees by pregnancy. While lymphocytes with complement receptors appear to be unaffected, those with receptors for AggIgG are diminished. These findings would tend to fortify those of two previously published studies in which surface markers of peripheral blood lymphocytes were examined during pregnancy. While Gergely and his associates examined surface immunoglobulin (SIg) and receptors for SRBC on the lymphocytes of pregnant females, Brain *et al.* examined only for cells with SIg (7, 8). Both

I. Percents and Absolute Numbers of Lymphocyte Subpopulations in Peripheral Blood at 9:00 a.m. in Pregnant and Nonpregnant Females[a].

	Nonpregnant female controls	Pregnant females
'mphocyte count (per mm³)	2799 ± 213 (23)[b]	2444 ± 164 (28)
[c]	21.9 ± 1.3 (23)	13.1 ± 0.7[d] (23)
.ggIgG Count (per mm³)	614 ± 68	307 ± 28[d]
	13.6 ± 1.4 (13)	14.0 ± 0.9 (15)
:RL Count (per mm³)	357 ± 42	338 ± 39
	70.7 ± 1.7 (10)	77.0 ± 0.9[f] (15)
' lymphocyte Count	1727 ± 191	1900 ± 187

values expressed as mean ± standard error.
ıber of subjects tested given in parentheses.
ıphocytes with receptors for aggregated IgG.
: 0.001).
ıphocytes with receptors for complement.
: 0.01).

groups found these subpopulations to be in normal numbers.

The findings here are compatible with the hypothesis that the immunological changes which occur during pregnancy may be mediated, at least in part, by one or more of the following alterations in certain lymphocyte subpopulations: (i) A decrease in the total number of certain subpopulations throughout the body; (ii) A redistribution of certain subpopulations from peripheral blood to other organs; (iii) Certain subpopulations may remain in the peripheral blood but their receptors may be modified so that they are no longer detectable. Changes in pool size or in receptors alone, however, may not be directly related to changes in cell function. Therefore, to know the true significance of the findings here, they must be correlated with studies of cell function directly, i.e., antibody-dependent cell-mediated cytotoxicity or *in vitro* immunoglobulin synthesis to evaluate the functional capacity of cells with receptors for AggIgG (9–11).

Since the blood levels of the steroid sex hormones (estrogen, progesterone, and possibly other steroidal hormones) rise in pregnancy, attempts have been made with some success by many investigators to reproduce the immunologic abnormalities of pregnancy by the administration of these hormones to nonpregnant subjects. Whether the changes seen in lymphocyte receptors during pregnancy can be reproduced by the administration of steroidal sex hormones is presently under investigation in our laboratory.

Summary. Peripheral blood lymphocytes from pregnant and nonpregnant females were studied for the presence of the following surface receptors: (i) Receptor for heat-aggregated human IgG (AggIgG); (ii) Receptor(s) for complement components; (iii) Receptor for sheep red blood cells (SRBC).

Absolute numbers of lymphocytes with receptors for complement and those with receptors for SRBC were present in equal numbers in both groups. However, in the pregnant females there was a significantly lower number of lymphocytes with receptors for AggIgG. It is hypothesized that some of the immunological changes which occur during pregnancy may be mediated partly through changes in the total numbers of certain lymphocyte subpopulations.

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The Contribution of the Distal Gastrointestinal Tract to Glucagon-like Immunoreactivity Secretion in the Rat (39464)

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glucagon-like immunoreactivity
s released after large enteric loads of
(1–3), fats (5), and salts (4) and it
erally been assumed that its function
ed to the disposal of these absorbed
ts. Studies of its distribution in the
of the gastrointestinal tract, how-
now that the highest concentrations
nd in the distal portions of the tract
n the dog (9) and the cat[2] the release
occurs from the ileum and colon,
ively, but not from the jejunum.
seems unlikely that a hormone in-
primarily with nutrients absorbed
he proximal gut would be secreted
by the distal gut, it was decided that
investigation of the role of the distal
e in GLI secretion was merited. The
of the study reported here was to
e the contribution of the ileum and
glucose stimulated GLI secretion in
common laboratory species, the rat.

ods. Thirty rats weighing 400–500 g
udied after an overnight fast. Anes-
as induced by an intraperitoneal in-
of sodium pentobarbital (50 mg/kg).
otomy was performed. In studies in
he entire gut or only the upper gut
be stimulated, a ligature was placed
the pylorus to prevent reflux and a
ucose solution was instilled over a 5-
riod directly into the duodenum
a 25-gauge needle. The volume ad-
ed was adjusted to deliver 2 g of
kg body weight. In studies in which
upper gut was to be stimulated, a
was also placed 45 cm proximal to
um (the approximate mid point of
num-ileum) and a drain was inserted on the proximal side. In studies in which only the lower gut was to be stimulated, the preparation was the same as for upper gut stimulation except that the glucose was instilled directly into the ileum. Blood samples were collected from a polyethylene cannula inserted in a femoral artery. Samples were collected just prior to and at 30-min intervals for 90 min after the introduction of glucose into the gut lumen. After immediately removing the plasma from each sample, the red blood cells were diluted 1:2 with normal saline and returned to the rat through a polyethylene cannula in a femoral vein. In control experiments the animal was prepared as for stimulation of the entire gut except that nothing was introduced into the lumen during sample collection. A 45-min period was allowed to pass between the time all cannulas, drains, and ligatures were placed and the start of the experiment. The animal's body temperature was maintained at 37–38° with the aid of a heating pad.

Blood samples of 1 ml volume for GLI determination were collected by syringe and placed into chilled 10 × 75-mm glass tubes containing 0.05 ml of Trasylol (FBA Pharmaceuticals, New York, New York) and 0.01 ml of sodium heparin (50 units). The samples were centrifuged immediately at 4°. The resulting plasma was stored at −20° until analyzed.

Acid-ethanol extracts of whole gut wall for assay of GLI were prepared from pooled tissues of four unfasted rats. Segments of midduodenum, midjejunum, midileum, midcolon, and pancreas were excised immediately after each rat was killed by decapitation. The tissues were rinsed in cold normal saline, blotted lightly and stored at −20° until extracted and purified by the method of Kenny (10).

The GLI concentration of samples of plasma or intestinal extracts was measured by radioimmunoassay (11) using a nonspe-

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ddress: Division of Endocrinology B-5, Har-
ral Hospital, 1000 W Carson St, Torrance,
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cific glucagon antiserum raised in rabbits against beef-pork glucagon coupled to polyvinylpyrrolidone (12). Free glucagon was separated from antibody-bound glucagon with dextran coated charcoal (13). Beef-pork recrystallized glucagon (a gift of Dr. Mary Root, The Lilly Research Laboratories, Indianapolis, Indiana) was used for standards and for preparation of [^{125}I]glucagon by the chloramine T method of Greenwood and Hunter (14). In this assay system dilution of gut extracts and dilution of pancreatic glucagon gave parallel inhibition curves; dilution of serum, however, did not. Therefore, all serum samples were assayed at the same dilution (1:6).

The statistical significance of GLI responses to glucose stimulation was determined by Student's t test applied to the difference between the response of an experimental group and that of the control group or another experimental group at designated times after the introduction of glucose. Until purified gut GLI is available for use in the radioimmunoassay, immunoassays of gut GLI using pancreatic glucagon as standard are useful only for revealing relative GLI levels, not absolute concentrations. Therefore, GLI responses here are expressed as percent of each animal's basal GLI concentration. The mean (±SEM) basal GLI concentration for all experiments was 608 ± 39 pg/ml.

Results. The concentrations of GLI in tissues of the gastrointestinal tract of the rat are shown in Table I. The lowest concentration was 14.4 ng/g wet weight in the duodenum. The jejunum had 77.6 ng/g. The highest gut concentrations were in the ileum, 213 ng/g, and the colon, 190 ng/g. The pancreas contained 798 ng/g.

The peripheral plasma GLI responses to the intraduodenal administration of 20% glucose, 2 g/kg, are shown in Fig. 1. When the entire gut was exposed to glucose, plasma GLI increased to a mean of 173% of basal at 30 min after glucose introduction, and remained elevated for the duration of the experiment. The response was statistically significant at all times tested ($p < 0.05$ at 30 min, $p < 0.02$ at 60 min, $p < 0.005$ at 90 min). When, however, the ileum and colon were excluded from contact with the glucose solution by a ligature and a drain placed at the midjejunum-ileum, peripheral plasma GLI increased to a mean of only 138% of basal at 30 min, remained at this level at 60 min, and then decreased slightly to 130% of basal at 90 min. The GLI response was still, however, significantly different from the control group at all times tested ($p < 0.025$ at 30 min, $p < 0.05$ at 60 min, and $p < 0.02$ at 90 min).

Since it appeared that over half of the GLI rise seen after intraduodenal glucose resulted from stimulation of the distal gut, the effect of intraluminal glucose in only the distal gut was examined. These results are shown in Fig. 2. Following the introduction into the ileum of 20% glucose, 2 g/kg, the peripheral plasma GLI concentration increased to a maximum of 163% of basal at 60 min. The response was statistically significant at all times tested ($p < 0.05$ at 30, $p < 0.01$ at 60, and $p < 0.01$ at 90 min). The GLI response to glucose in only the upper gut shown in Fig. 1 is repeated in Fig. 2 to facilitate comparison with the response to

TABLE I. CONCENTRATION OF GLI IN GASTROINTESTINAL TISSUES OF THE RAT.[a]

Tissue	[GLI] ng/g wet weight
duodenum	14.4
jejunum	77.6
ileum	213
colon	190
pancreas	798

[a] Each sample analyzed was an acid ethanol extract of 1 g of tissue obtained by pooling 0.25 g pieces of tissue from four rats. Mean of four determinations on each sample.

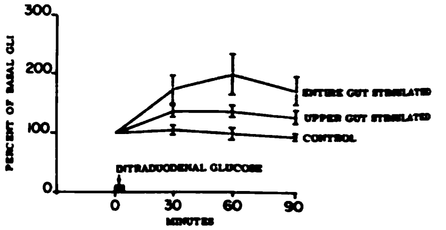

FIG. 1. Mean responses of peripheral plasma GLI concentrations, expressed as percent of basal concentration, to no stimulation (control, $N = 4$), to stimulation of only the upper gut ($N = 10$), and to stimulation of the entire gut ($N = 8$) by the intraduodenal instillation of 2 g/kg body weight of 20% glucose. The vertical lines indicate 1 SEM.

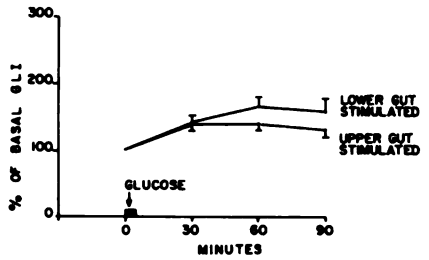

FIG. 2. Mean responses of peripheral plasma GLI concentration, expressed as percent of basal concentration, to stimulation of only the upper gut ($N = 10$) and only the lower gut ($N = 8$) by the intraluminal instillation of 2 g/kg body weight of 20% glucose. The vertical lines indicate 1 SEM.

glucose in only the lower gut. Glucose in the lower bowel resulted in a slightly greater GLI release at 60 and 90 min than did glucose in the upper bowel but the difference was not statistically significant.

Discussion. This study shows that GLI is present throughout the intestinal tract of the rat with the highest concentrations occurring in the distal portion of it. A similar distribution has been reported by Unger *et al.* (15) and Schopman *et al.* (8) in man, by Buchanan *et al.* (17) in the dog, by Bloom and Bryant (7) in the rat and baboon, and by Bataille *et al.* (16) in the pig. Immunofluorescent staining techniques show that enteroglucagon producing cells exist primarily in the ileum of man (6) although the colon was not examined in this study. The rat, therefore, seems to be a typical species with reference to its pattern of distribution of GLI in tissue.

This study further shows that in the rat, as in man (1, 2) and the dog (3, 17), enteric glucose stimulates the secretion of GLI *in vivo*. This finding supports the results of Luyckx and Lefebvre (18) who found release of GLI from rat jejunum incubated with 20% glucose *in vitro*. Zandomeneghi and Buchanan (19) also demonstrated GLI release from rat small intestine incubated with glucose solutions *in vitro*. The results reported here indicate that both the proximal and distal intestine are involved in glucose stimulated GLI secretion, with each contributing approximately equal portions of the total response when large glucose loads are introduced into the duodenum.

When glucose was placed directly into the ileum so that only the ileum and colon were exposed to it the observed GLI response was essentially the same as that seen when only the duodenum and jejunum were stimulated by intraluminal glucose. The distal and proximal gut of the rat therefore release equal amounts of GLI when both are exposed to the same glucose load. This observation is somewhat surprising in view of the fact that greater concentrations of GLI are extracted from the ileum and colon than from the duodenum or jejunum. Matsuyama and Foa (9) have shown that, in the dog, stimulation of ileal loops with 5% glucose solutions elicited a marked rise in GLI in the venous effluent of the loop, whereas identical stimulation of jejunal loops produced no significant change in GLI concentration. In the cat it has been shown that the colon secretes GLI in response to intraluminal glucose but the jejunum and ilem do not.[2]

In three different species, therefore, GLI secretion from the distal intestinal tract equals or exceeds that from the proximal tract when both parts are equivalently stimulated. If a similar situation exists in man, it could explain the hypersecretion of gut GLI following oral glucose reported in gastrectomized persons (20–22). It could also explain the excessive GLI responses to alimentary glucose described in patients with dumping syndrome (23, 24). In both types of subjects a larger portion of an ingested glucose load would be expected to reach the distal intestine than would reach it in normal persons. Bloom (25) in fact has hypothesized that gut GLI release from the ileum may explain the period of hypomotility which follows the initial rapid transport of substances through the small bowel in subjects with dumping syndrome. That gut GLI may cause hypomotility was suggested by the pronounced stasis of the large and small bowel seen in a patient with an enteroglucagon secreting tumor (26, 27). The stasis remitted when the tumor was removed. The physiological role of gut GLI is purely speculative at this point. GLI's presence in and secretion from the distal gut, however, are factors which should be considered in future research on this problem.

Summary. Radioimmunoassay of acid ethanol extracts of the rat intestinal tract showed the presence of glucagon-like immunoreactivity (GLI) from the duodenum to the colon with maximal concentrations in the ileum and colon. Twenty percent glucose instilled in the duodenum at a dose of 2 g/kg body weight stimulated a twofold increase in peripheral plasma GLI concentration. When the instilled glucose load was restricted to only the duodenum and jejunum, or to only the ileum and colon, the increase in peripheral plasma GLI was approximately half that seen when the entire gut was exposed to the glucose. It is concluded that the distal gut as well as the proximal gut releases GLI in the rat.

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roup Composition of Membrane Phospholipids in Mammary Tissues and Carcinoma Induced by Dimethylbenz(*a*)anthracene[1] (39465)

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ent years, considerable effort has
cted toward understanding the role
acids in membrane functions (1).
ne-bound hormone receptors, for
the prolactin receptor molecule,
n reported to contain a phospho-
tion (2). Changes in fatty acid com-
may alter the structural integrity of
e, and possibly also affect the
ctivity of membrane-bound recep-
of hormones. These changes may
lify the transport of critical nutri-
le the cell, thereby promoting the
tion of a neoplastic state (3). Re-
m our laboratory (4) and from
) showed that acyl group composi-
dividual phospholipids in subcellu-
ions of human brain tumors ex-
pattern different from the corre-
fractions of normal brain tissues.
aring hepatomas with host liver tis-
er investigators (6, 7), also demon-
anges in the acyl group patterns of
The present investigation was un-
to see whether similar changes
r in mammary tumors of rats in-
DMBA as compared to normal
sues. Since DMBA-tumors are es-
rolactin dependent (8), it may be
to establish a relationship between
onal effect on tumor growth and
n composition of phospholipids in
nors.

ls and Methods. Induction of tu-
mors were induced in 50-day-old
Dawley rats (Holtzman Co., Madi-
) by a single intragastric feeding of
16 mg of DMBA (Sigma Chemical Co., St Louis, Mo.), suspended in sesame oil. In about 6 weeks, tumors began to develop. The presence of estrogen receptors, which is a characteristic of estrogen-dependent tissues (9, 10), was detected in all the tumors in this study. These tumors were also identified histologically as adenocarcinoma. Portions of the tumors were frozen for lipid analysis. Breast tissues were isolated from tumor-bearing rats either contralaterally to the tumors or, if ipsilaterally, at a remote distance from the tumors.

After the connective tissues, skin or visible necrotic areas were removed, and the tumors or normal breast tissues were weighed and minced with scissors. A 4-vol to tumor-weight of Tris-EDTA buffer (10 m*M* Tris and 1.5 m*M* EDTA, pH 7.5) was added. The suspension was homogenized with a polytron PT-10 tissue disintegrator (Brinkmann Instrument, Westbury, N.Y.) for two 15-sec periods with a 30-sec cooling interval. The resulting homogenate was centrifuged for 15 min at 3500*g* to remove cellular debris and red blood cells. The supernatant solution was used for the extraction of lipids.

Analysis of membrane lipids. Tissue lipids were extracted with chloroform–methanol, 2:1 (v/v) (11). Neutral glycerides were separated from phosphoglycerides by Unisil column elution (12). Neutral glycerides and other nonpolar lipid materials were eluted by passing 50 ml of chloroform through a column packed with 2 g of Unisil. Phospholipids and other polar lipids were eluted with 100 ml of methanol. Individual neutral glycerides and phosphoglycerides were further separated by tlc. The thin-layer plates were prepared with Silica gel G suspended in 0.01 *M* Na_2CO_3 (Brinkmann Instruments, Westbury, N.Y.). Triglycerides were separated by one-dimensional tlc, using hex-

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iations used: Diacyl-GPC, diacyl-glycero-
choline; diacyl-GPE, diacyl-glycerophos-
olamine; diacyl-GPI, diacyl-glycerophos-
tol; diacyl-GPS, diacyl-glycerophosphoryl-
thin-layer chromatography; glc, gas-liquid
aphy; DMBA, 7,12-dimethylbenz(a)an-

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ed.

ane:diethyl ether:15 *N* ammonium hydroxide 70:30:0.1 (v/v) as solvent for development. Phospholipids were subjected to separation–reaction–separation by tlc as described by Horrocks and Sun (11), except that the solvent system for the second dimension was modified (13). After solvent development, lipid spots were visualized by exposing the thin-layer plates to iodine vapor, as in the case to be used for phosphorus assay. When the lipid spots were required for fatty acid analysis, the thin-layer plates were sprayed with ethanolic 2′,7′-dichlorofluorescein. Individual lipid spots were scraped into test tubes and the amount of lipid-phosphorus was determined according to Gottfried (14). For the analysis of acyl group composition of the phospholipids and triglycerides, lipids were subjected to alkaline methanolysis (15). Methyl esters of fatty acid were analyzed by a gas chromatograph fitted with a digital integrator (Hewlett Packard). Repeated analyses of the same sample showed good reproducibility to within 5% variation of the peak area.

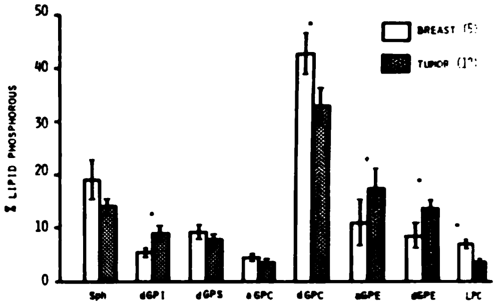

FIG. 1.. Membrane phospholipid composition of DMBA-tumor and mammary tissues. Methods of membrane preparation and quantitation of phospholipids were described in the text. Values (mean ± SD) are expressed as percentages of total lipid phosphorus content. Ten tumor samples and five mammary glands of the same tumor-bearing animals were analyzed, respectively. Only four tumors have measurable LPC fractions. Abbreviations: Sph, sphingomyelin; dGPI, diacyl-GPI, dGPS, diacyl-GPS; aGPC, alkenylacyl-GPC; dGPC, diacyl-GPC; aGPE, alkenylacyl-GPE; dGPE, diacyl-GPE, LPC, monoacyl-GPC; * and † show statistical significance of $p < 0.001$ and 0.005, respectively, by the student's *t* test (tumor vs control).

Results. Compositions of phospholipids in breast tissues and tumors from DMBA-treated animals are shown in Fig. 1. Approximately 40% of the phospholipids of the control breast tissues were diacyl-GPC. Sphingomyelin, alkenylacyl-GPE and diacyl-GPE each constituting about 10 to 20%. Cardiolipins were present in breast tissue and tumors in small amounts (2–5%) but were not analyzed because the lipid spot was not completely separated from the free fatty acid spot in the thin-layer system. In the DMBA-tumors there was an increase in the proportion of alkenylacyl-GPE, diacyl-GPE, and diacyl-GPI and a decrease in the proportion of diacyl-GPC as compared to controls. A small proportion of monoacyl-GPC was also present in some breast tissues and tumors, but there was no apparent alteration in the proportion of this phosphoglyceride in the tumor tissue. The value of the ratio diacyl-GPE/diacyl-GPC of tumors was about two times higher than that of the normal breast tissues.

Phosphoglycerides of breast tissues and tumors were characterized by their distinct patterns of fatty acids (Fig. 2). Acyl groups of diacyl-GPC were high in 16:1 and 18:1; diacyl-GPE in 18:0, 18:1, and 20:4; diacyl-GPI in 18:0 and 20:4; and diacyl-GPS in 18:0 and 18:1. In contrast, acyl groups of alkenylacyl-GPE contained mainly 20:4. Acyl group composition of phosphoglycerides in adenocarcinoma had a higher proportion of 18:1, and a lower proportion of 18:2 than breast tissues. Moreover, an increase in the proportion of 16:0 was also observed in some phosphoglycerides of tumors. There was no apparent change in the proportion of 20:4 (*n*-6) among other phosphoglycerides (Fig. 2A–C) except for a slight decrease of 20:4 (*n*-6) in diacyl-GPI in tumors (Fig. 2D). Although quantitative changes in fatty acids were demonstrated, each individual phospholipid in tumor tissue appeared to maintain the same distinct acyl group profile as in normal tissue.

About 60% of the fatty acids of alkenylacyl-GPE was 20:4 (*n*-6), but the proportion of this fatty acid was not altered in the neoplastic tissue (Fig. 3A). Consequently, acyl group composition of alkenylacyl-GPE in tumors remained essentially the same as in breast tissues. Triglycerides of breast tissues and tumors were high in 16:0, 18:1, and 18:2 fatty acids (Fig. 3B). In contrast to phosphoglycerides, there was no remarkable variation in acyl group pattern of triglyc-

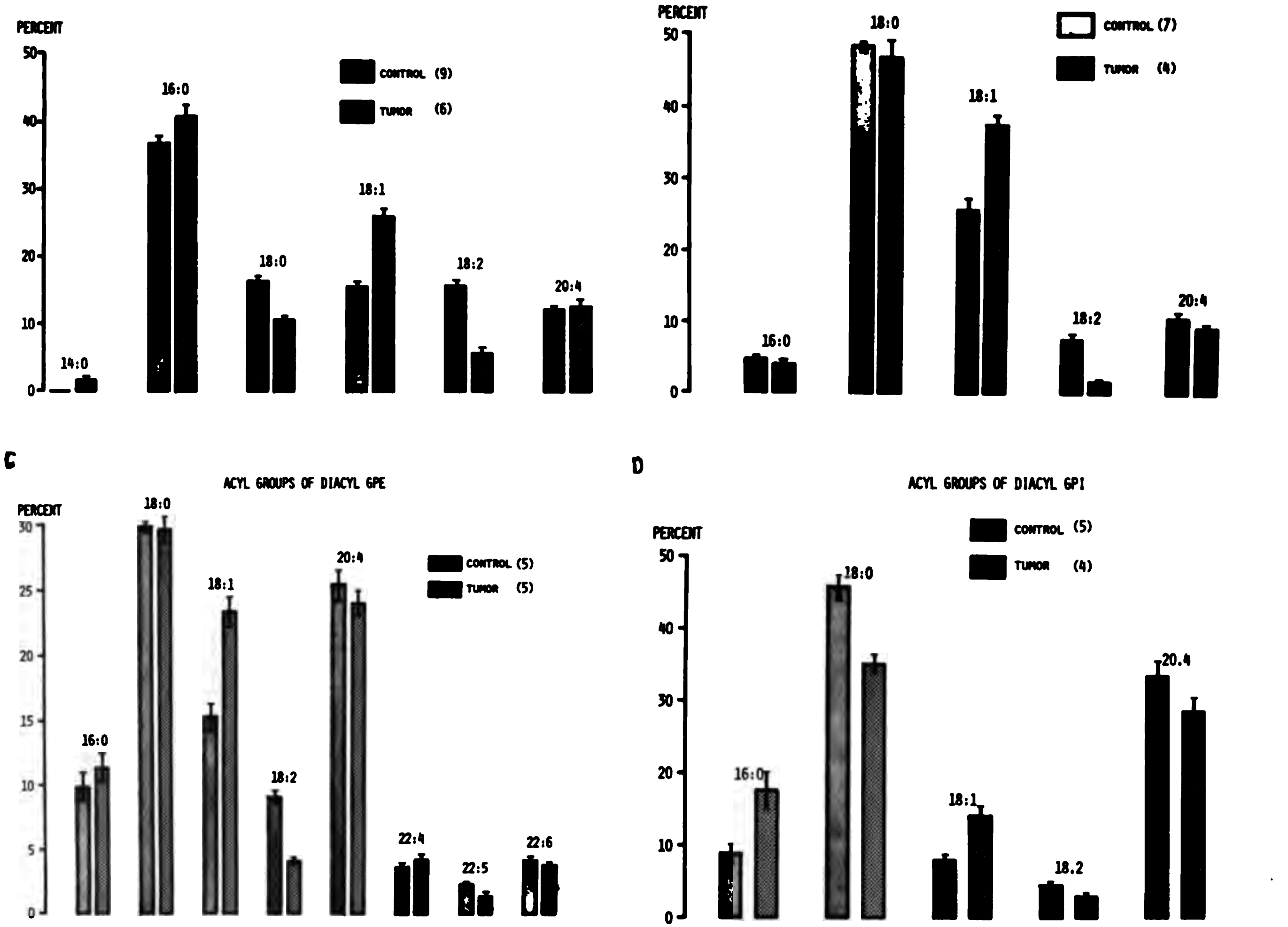

FIG. 2. The acyl group compositions of individual phosphoglycerides in DMBA-tumor and breast tissues. Isolation of membrane phospholipids and determination of fatty acid by glc are as described in materials and methods. Values (mean ± SEM) are expressed as weight percents according to peak areas of glc. The number of determinations are as indicated in parentheses of the chart.

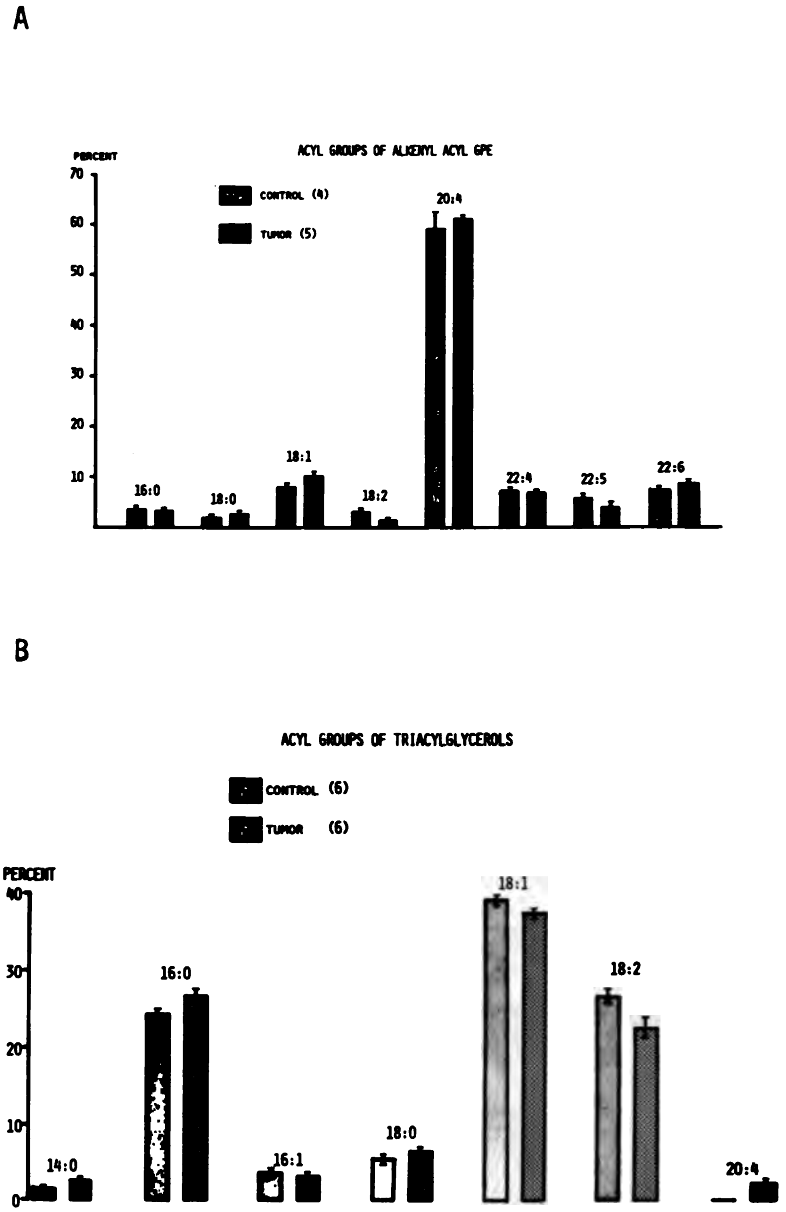

FIG. 3. The acyl group compositions of alkenylacyl-GPE (A) and of triglycerides (B) in tumors and breast tissues. See Fig. 2 for details.

erides between breast and tumor tissues.

When the relative amounts of 20:4 to 18:2 of the various phosphoglycerides for breast and tumor tissues were evaluated (Table I), an elevation of the ratio of phosphoglycerides for tumors was observed. The increase in ratio was accounted for largely by the decrease in the proportion of 18:2 in tumors.

Analyses of phospholipids and fatty acids were also performed with normal breast tissues obtained from rats without DMBA treatment and at various periods during pregnancy and lactation (16). These studies showed changes in phospholipid and acyl group compositions in breast tissue during different physiological states. However, the lipid composition between breast tissue

.E I. Ratio of 20:4/18:2 in Breast Tissues
nd Tumors of DMBA Treated Rats.[a]

	dGPI	aGPE	dGPC	dGPE	dGPS
tissue	7.6	19.9	0.8	2.8	1.3
.-tumor	9.8	47.8	2.2	5.8	5.1

tio of 20:4/18:2 was calculated from the fatty
ntent of each individual phospholipids as shown
2 and 3.

normal rats and from DMBA-treated
vas similar. Hence, breast tissue of
A-treated animals was selected as con-
ssue for comparison.

cussion. Changes in phospholipids
>e a common abnormality of cellular
>ranes in transformed cells (17). Dif-
:es in composition of phospholipids
en DMBA-tumors and control breast
s were demonstrated in the present
. Tumor tissue had a decrease in dia-
PC and an increase in diacyl-GPE, al-
acyl-GPE and, diacyl-GPI. Since com-
ıns were made on pieces of normal and
tissues and not on specific cell types,
ossible that the normal and neoplastic
samples contained different propor-
of blood and connective tissue. There-
some of the observed changes could
t tissue compositional differences
than those due to mammary tumors,
. However, histological examinations
revealed that the tumor specimens
composed primarily of adenocarci-
cells, suggesting phospholipid and
acid changes were due to the cancer

ferences in acyl group profiles of indi-
phospholipids between DMBA-tu-
and breast tissues were demonstrated
; study. Although both tissue and tu-
contained a high proportion of triglyc-
, fatty acid changes in tumors were
specific for phospholipids than for tri-
ides. The most marked change was
:crease in the proportion of 18:2 in the
hoglycerides of tumor. Since 18:2 is
ential fatty acid which is supplied ex-
usly to the cells rather than synthe-
by the cells, reduction of 18:2 in phos-
ids may reflect a decrease in the utili-
of this fatty acid as a structural com-
t for cellular membrane. In the pres-
periment, the decrease in the proportion of 18:2 was compensated largely by the increase of 18:1. This condition was similar to that observed during chronic deficiency of essential fatty acid (18), except that the characteristic increase of 20:3 (*n*-9) in tissues was not noted in breast tumors. Possibly there was an inadequate supply of the nutrient fatty acids in tumor cells, resulting in changes similar to that of a localized deficiency of essential fatty acid.

Changes in acyl group composition of phosphoglycerides have been reported for a number of hepatomas in comparison to fatty acid composition of the respective normal tissues (6, 7). In most tumors examined, there was an increase in the proportion of 18:1 in the major phosphoglycerides but a decrease of 18:2 was reported only in Morris Hepatoma (5123C) (7). In general, a decrease in the proportion of 20:4 (*n*-6) was also noted in these tumors. Brain tumors have been reported to exhibit an increase in 20:4 (*n*-6) and a decrease in 22:6 (*n*-3) when compared to normal brain tissues (4). The cause for acyl group changes among different tumors is not known. However, these changes may be related to many cellular abnormalities since the acyl groups of phosphoglycerides are not only structural ingredients of membranes but are also important in regulating different cellular functions.

The turnover of 18:2 with respect to its conversion to 20:4 may have been altered during neoplastic transformation. Since 20:4 (*n*-6) is a precursor for the biosynthesis of prostaglandins F_2 and E_2 (19), the decrease of 18:2 and 20:4 in diacyl-GPI of tumor might reflect a more rapid biosynthesis of prostaglandin. Indeed, Tan *et al.* (20) have recently demonstrated a higher concentration of prostaglandin E_2 and a relatively more rapid conversion of labeled arachidonic acid to prostaglandin in DMBA-tumors than in normal breast tissue.

In conclusion, characteristic phospholipid profiles and their side chain fatty acids in breast tissues were altered in neoplasm. Changes in fatty acid composition were similar among phospholipids. There was no apparent change in fatty acids in triglycerides. It is suggested that an altered rate of metabolism of the essential fatty acid, 18:2, may have occurred in tumors. Modifications of phospholipids in the membrane could, in turn, contribute to abnormal functions or differentiation of cells.

Summary. Phospholipids and their acyl group composition in mammary adenocarcinomas and mammary tissue of the same tumor-bearing animals were investigated. Breast adenocarcinoma induced by dimethylbenz(*a*)anthracene exhibited a phospholipid pattern which was different from that of the mammary tissue. Tumor phospholipids had higher proportions of diacyl-GPI, diacyl-GPE, and alkenylacyl-GPE and a lower proportion of diacyl-GPC than the controls. The acyl groups of most phospholipids in tumors showed a marked increase in the proportion of 18:1 and a decrease in the proportion of 18:2. The fatty acid composition of plasmalogen and triglyceride, however, remained unchanged. In spite of the decrease in the proportion of 18:2, there was no apparent difference in the proportion of 20:4 in most of the phosphoglycerides; however, a significant decrease in this fatty acid was noted in diacyl-GPI. Results of this study demonstrated that the membrane phospholipids of mammary adenocarcinoma were altered in respect to acyl group composition. Changes in physical properties of the cell membrane, in turn, could lead to abnormal manifestation of membrane regulated events in tumor cells.

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A Study of Taste and Smell of Heavy Water (99.8%) in Rats[1] (39466)

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s remarkable hydrogen isotope, deute- oxide, constitutes one part in 5000 of linary water no matter where found in orld. How and when it ever became orated in ordinary water is not defi- known. But it has been assumed that ater first appeared at the time of the "Bang" of the universe (1).

mically, deuterium differs from hy- n in that it has a mass of two. Physi- heavy water differs from ordinary wa- a number of properties; for instance, ling to Thompson (2) it is denser; it s, melts, and boils at higher tempera- it is more viscous; it is a poorer sol- It looks, however, exactly like ordi- vater.

the present experiments rats were a choice of distilled water and heavy (99.8%) to determine whether they ize a difference between them, and if what basis—taste, smell, or other teristics?

hod. Cages used for these experi- were made of 1-cm wire mesh 19 × 25 cm (3). Graduated inverted 100-ml s fitted into holes placed at symmetri- sitions at either side on the front of the Food was available *ad lib.*[2]

eliminate differences in temperature, es of distilled water and heavy water cept at room temperature at all times.

rats drink heavy water (99.8%) as as distilled water? For this control ex- ent only one bottle was used. For 15- ys this bottle was filled with distilled . Records were made daily of the intake. When the intake had reached a fairly constant level, heavy water (99.8%) replaced distilled water and daily records were made as before.

Eighteen rats were used.

Results. In Fig. 1A for a rat, ordinates show fluid intake in milliliters; abscissas show time in days. Intake of distilled water was measured for 22 days. During the last 10 days the intake averaged 27 ml. Then heavy water replaced distilled water. On the first day the rat drank the heavy water just as freely as it had the distilled water. From then on it drank progressively less each day until its death on the twelfth day. Figure 1B shows the record of a rat that on the first day drank more heavy water than it had previously been drinking distilled water. It drank progressively less each day and died on the seventh day. The 16 other rats gave similar records. The 18 rats lived an average of 14.0 days on heavy water. On first exposure the rats certainly did not show any aversion to heavy water.

To determine whether rats can tell the difference between heavy water and distilled water and at what concentration. For this experiment I used the "preference threshold" technique which had been widely used in the study of reactions of rats to various nutritive substances (sodium chloride (3), five common sugars (4), alcohol (5)) and toxic substances (arsenic trioxide (6), phenylthiocarbamide (7), thallium sulfate (6)). In all instances, this technique gave sharply defined recognition thresholds.

For this technique the afore mentioned rectangular cage held two graduated inverted 100-ml bottles (made as nearly identical as possible), one at each side in symmetrical positions. These two bottles were filled with distilled water each day until the intake from each one had reached a fairly constant level. In most instances a rat consistently drinks more from one bottle than

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rried out under grants from the National Insti- Mental Health (MH00576-22) and National Foundation (GB-23367); presented in part at a at the Department of Psychology at Princeton, rsey, February 28, 1973.

aham flour, 72.5%; skimmed milk powder, casein, 10.0%; butter, 5.0%; calcium carbon- %; sodium chloride, 1.0%.

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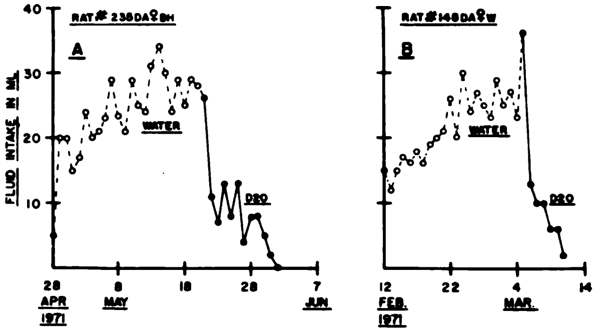

FIG. 1. Daily intake of distilled water for two rats; then restriction of intake to heavy water (99.8%).

the other. Then a subliminal concentration of a solution of the substance to be tested, if nutritive is placed in the bottle from which the rat had been drinking the smaller amounts, and if toxic in the bottle with the higher intake of distilled water. In either instance, concentrations of solution are increased by small steps at regular intervals (usually one day) until the intake levels definitely show that the rat has recognized presence of the test substance.

For all the many substances tested this technique gave clear-cut consistent results, with only narrow margins of individual differences.

For the present experiment (undertaken in 1970 at once after discovering that rats do not survive on heavy water) heavy water in a 5% concentration was placed in the bottle from which the rat had been drinking the larger amounts of distilled water. Then, every other day the concentrations were increased by 5% until they reached 99.8%.

Nine adult rats were used for this experiment. Records were made daily of intake from the two bottles; the rats were weighed every second day.

It must be stated here at once that the wide range of individual differences of reactions of the rats to heavy water make it impossible to give an average curve as was always possible with the previously tested substances.

Figure 2A shows the records for one of the rats that did not recognize a difference between heavy water and distilled water. Ordinates show daily intake in milliliters and body weight in grams; abscissas show time in days; the stepograph shows concentrations of heavy water from 5 to 99.8% (100). Intake of distilled water and heavy water fluctuated up and down right up to the end. On concentrations above 50% the rat began to lose weight showing that it was ingesting harmful amounts of heavy water. Interchanging positions of the bottles produced only a temporary decrease in intake of heavy water. Three other rats showed similar eratic records with no definite recognition of heavy water.

Figure 2B shows the record of a rat that drank only heavy water up to 80%; then after interchanging positions of bottles drank less heavy water for 3 days; then drank very large amounts and as a result died. It had lost weight at a rapid rate for the previous 7 days.

Figure 3A shows the record of a rat that after marked fluctuations in intake of distilled water and heavy water finally made a clear-cut recognition. It continued to gain weight throughout. Two other rats showed similar records. Figure 3B shows the only record that bears any resemblance to those obtained on the other substances. The rat recognized the heavy water solution in concentrations of 50% and above. It was then decided to give the rats a choice simply between heavy water (99.8%) and distilled water.

Are rats able to recognize heavy water when given a choice of distilled water and a 99.8% concentration from the very start? The technique was the same as before. The bottle from which the rat had been drinking the larger amounts was filled with heavy water (99.8%). Daily records of intake of distilled water and heavy water were then taken for several weeks.

The two bottles on each cage remained in

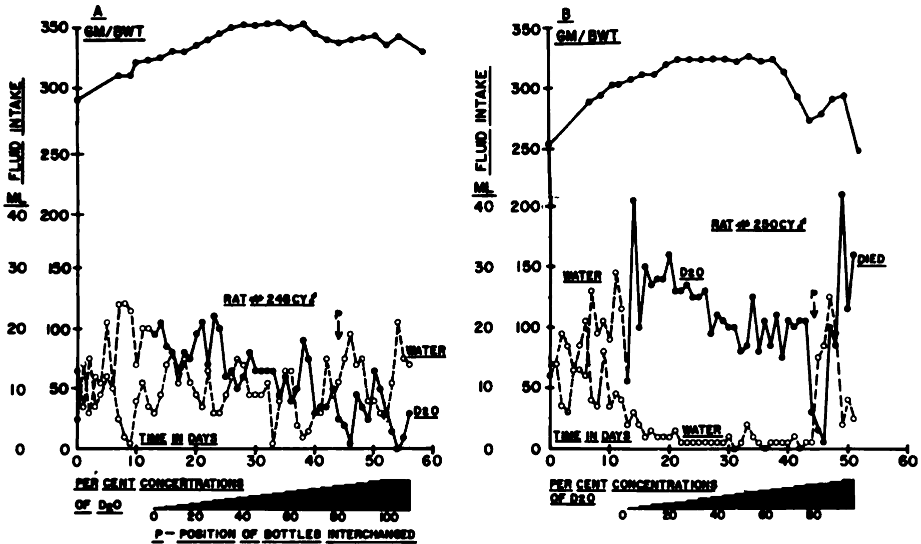

FIG. 2. Preference threshold records for two rats that had a choice of distilled water and heavy water in increasing concentrations (5% every other day) from 5 to 99.8%.

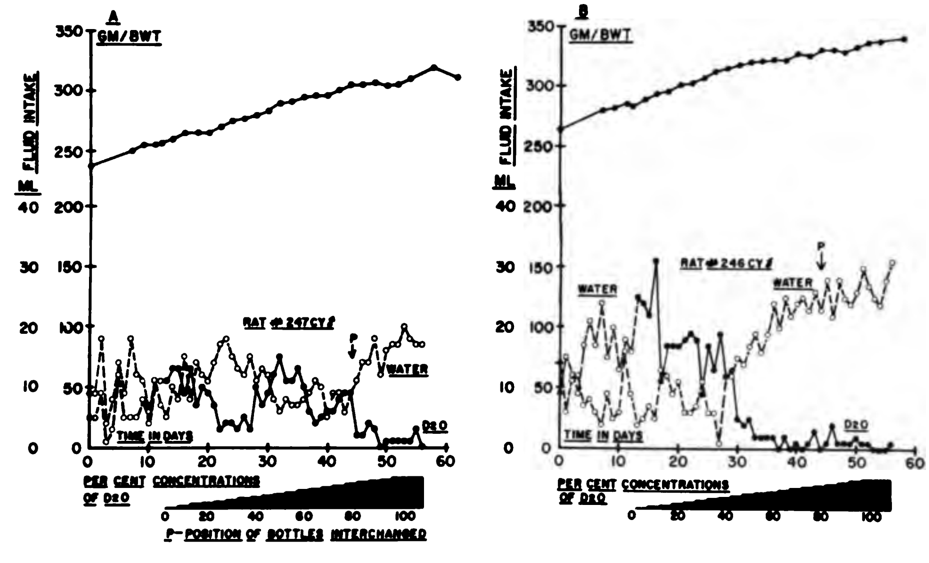

FIG. 3. Preference threshold records of two other rats.

the same position throughout the experiment unless otherwise specified.

Eighteen rats were used for this experiment.

Results. Figure 4A shows the record of one of these animals. On the first day this rat drank heavy water just as freely as it had been drinking distilled water. On the following days it practically stopped drinking heavy water but increased its distilled water intake to a normal level. After positions of the bottles were interchanged (P) the rat still refused to drink the heavy water. Here the rat definitely recognized the heavy water and almost showed an aversion to it. It gained weight at a constant rate throughout. Fourteen of the rats showed this type of record.

In view of the eratic unpredictable reactions of the nine rats in the "preference threshold" experiment, the fact that 14 out of 18 rats gave the Fig. 4A type of record

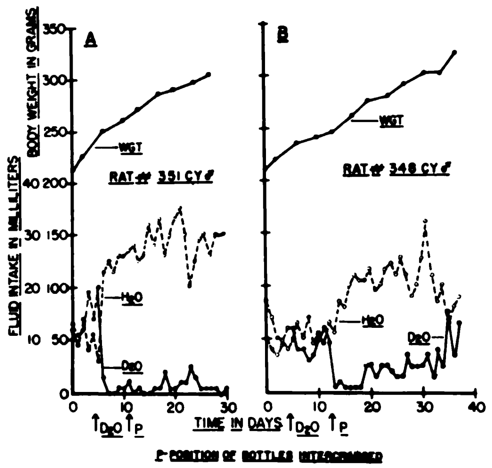

FIG. 4. Choice of distilled water and heavy water (99.8%) for two rats.

must be of considerable significance.

Figure 4B shows a different type of record. This rat started by drinking almost equal amounts of distilled water from the two bottles. For the first 8 days it drank heavy water and distilled water in nearly equal amounts. After interchanging positions (P) of the bottles it stopped drinking heavy water for 6 days and took larger amounts of distilled water. Then it drank progressively more heavy water, finally drinking almost equal amounts of each. Body weight, however, increased at a steady rate throughout, indicating that so far the rat had not ingested harmful amounts. Two rats showed this type of record. These records and those in Figs. 5A and B are probably further instances of eratic behavior seen in the "preference threshold" tests.

Figures 5A and B show still two other types of records. In Fig. 5A, positions of the bottles were interchanged every other day. This rat apparently drank enough heavy water on the first 2 days to produce a temporary aversion. After that it failed to differentiate. Position habits apparently made it drink fairly large amounts of heavy water every other day. However, it did not ingest enough heavy water to cause more than a very slight decrease in weight gain. In Fig. 5B the rat drank almost equal amounts of distilled water and heavy water at the start; then drank smaller amounts of heavy water. Changing of positions of bottles (P) did not *have any definite* effect on the fluid intake.

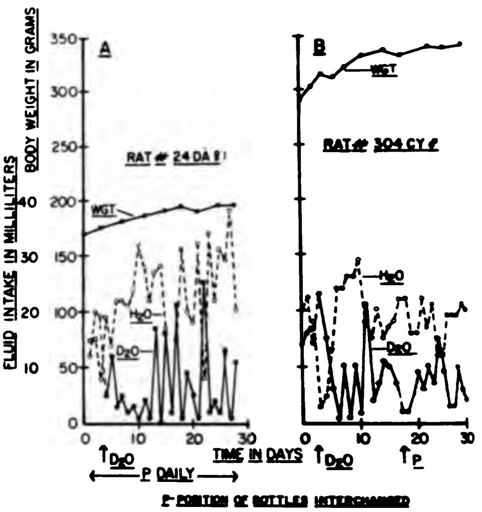

FIG. 5. Choice of distilled water and heavy water (99.8%) for two other rats.

Results of these experiments indicate that when on the first day the rats drank high amounts of heavy water and little or no distilled water, that they then developed a definite aversion to heavy water; whereas, when on the first few days they drank equal amounts of distilled water and heavy water, in some instances they did not make the distinction for 20–40 days or more.

This finding could mean that ingestion of high amounts of heavy water on the first day, that is when the rats drank it as freely as distilled water (i) they experienced untoward symptoms and (ii) they detected some characteristic of heavy water that made it possible for them to distinguish it from the distilled water and so to associate it with untoward symptoms.

What are the untoward symptoms? There is, of course, no way of determining what the rats experience. Apparently, they do not experience the untoward symptoms during the first 24 hr, since during this time they continued to drink heavy water as freely as distilled water. To throw light on this question determinations were made of heavy water content of the blood at various times after the start. Blood was taken at 8-hr intervals from the conjunctival sac in the corner of an eye with the method of Halpern and Pacaud (8).[3] Figure 6 shows curves

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[3] Dr. Josef Pitha, Department of Gerontology of the

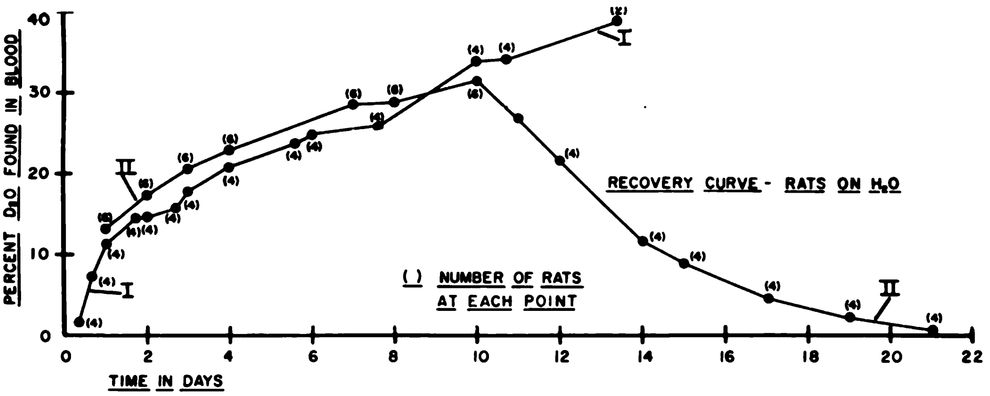

FIG. 6. Heavy water content of blood of rats whose fluid intake was restricted to heavy water (99.8%); and ter to distilled water.

rom two sets of readings. At the end of the irst 8 hr heavy water content had reached bout 7% and at end of the first day 12%, t the end of the second day 15–17%, and fter 10 days as much as 30%. We have no lea what symptoms these amounts of heavy ater produce. Previous experiments demnstrated that first signs of slowing of the 24 r clock occurred on the second day and it is kely that many organs showed very slight lowing effects (2). Various symptoms toether might suffice to produce untoward ymptoms enough to elicit the aversion to eavy water.

The following experiments were underaken to determine what characteristics of eavy water that the rats might associate ith the untoward symptoms.

Do rats taste heavy water? This experient was undertaken to determine whether he rats are able to taste heavy water. For his purpose taste nerves were cut in nine ats before the sharp differentiation shown n Fig. 4A was made; and in three rats ometimes afterwards.

The chorda tympani, glossopharyngeal, nd the pharyngeal branches of the tenth erves were cut or avulsed through expoures and methods described with Malone 9) in 1956. Taste buds in the fungiform, olliate, and circumvallate papillae degenerted after this operation.

Results. Figure 7A shows the record of ne of the rats in which the nerves were cut before the differentiation was made. It shows the intake of distilled water for 20 days after sectioning of the nerves. Heavy water was then placed in the bottle from which the rat had been drinking the larger amounts. On the first day, the rat drank heavy water as freely as distilled water. On the next day, however, it almost completely stopped drinking it, thus, demonstrating that the differentiation did not depend on the ability of rats to taste heavy water. The other eight rats showed the same results. Figure 7B shows that sectioning of the taste nerves after a clear cut differentiation had been made did not interfere with the rat's ability to recognize the heavy water. Interchanging positions of the bottles as well as interchanging of distilled water and heavy water without changing positions of the bottles did not alter the results. The other two rats gave the same results.

Are rats able to smell heavy water? This experiment was undertaken to determine whether rats are able to smell heavy water. For this purpose olfactory bulbs were removed in 16 rats before the differentiation was made between distilled water and heavy water, and were removed in six rats afterwards.

Results. Figure 8A shows the record of one of these rats. The olfactory bulbs were removed 8 days before start of the heavy water which was placed in the bottle from which the rat had been drinking the larger amounts of distilled water. This rat drank heavy water as freely as distilled water, not only on the first day, but on the 14 following days right up to the time of its death. It di

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ational Institute of Health in Baltimore and Dr. Cathrine Fenselau have my gratitude for many helpful ggestions and use of the spectrophotometer.

not, at any time, start to drink more distilled water. This record shows that this anosmic rat could no longer recognize the difference between heavy water and distilled water. Body weight showed a sharp decrease from the start with heavy water. Ten other anosmic rats showed this same result.

Figure 8B shows the record of an anosmic rat that continued to drink fairly large amounts of distilled water along with large amounts of heavy water. It lost some weight but did not die until 35 days after start of the heavy water. By that time its body weight had dropped far below the predicted level. It clearly did not differentiate between distilled water and heavy water. Figure 8C

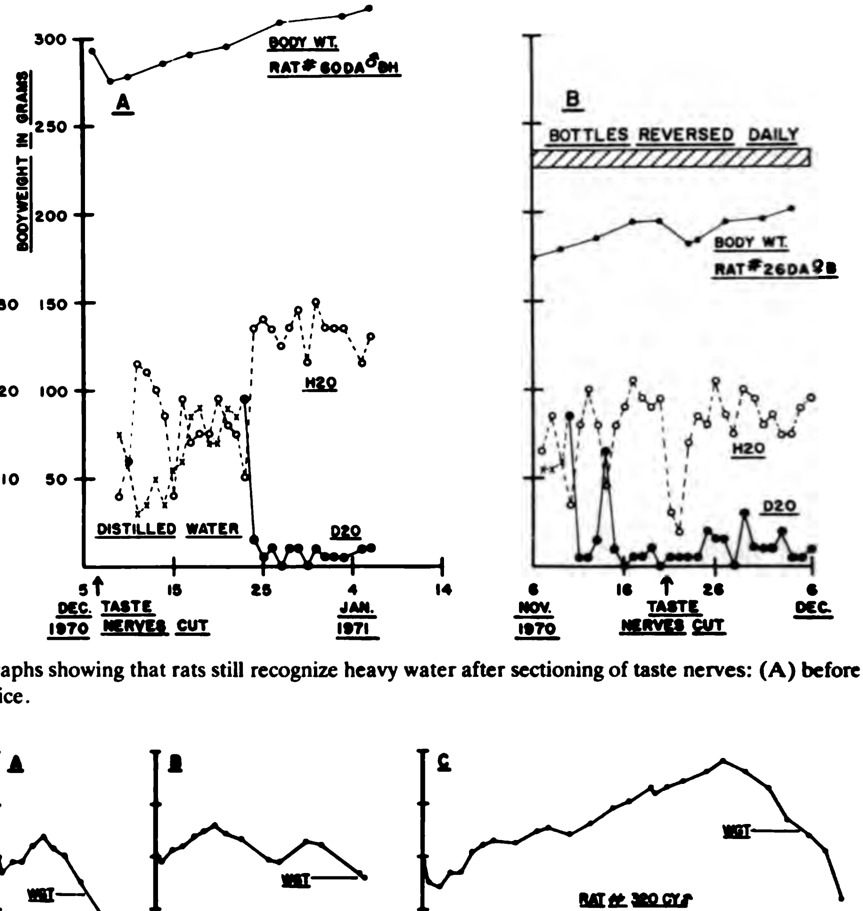

FIG. 7. Graphs showing that rats still recognize heavy water after sectioning of taste nerves: (A) before choice; (B) after choice.

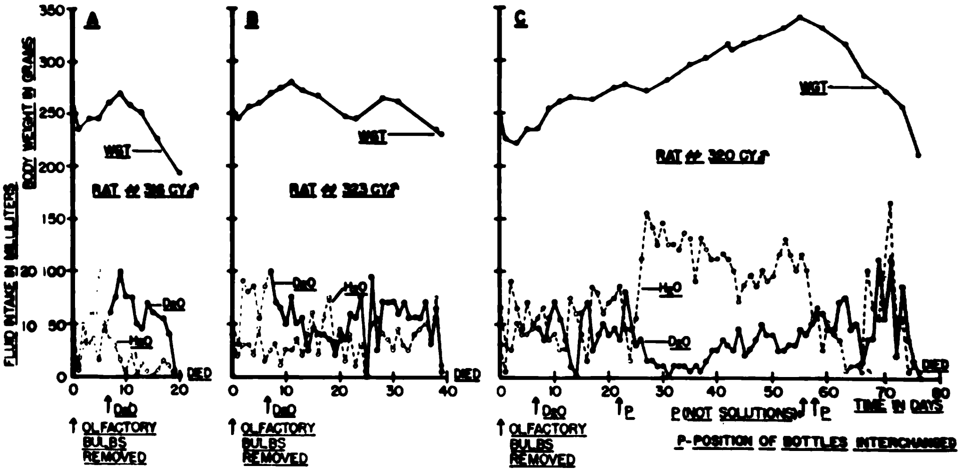

FIG. 8. Three graphs showing inability of rat to recognize heavy water (99.8%) after removal of olfactory bulbs.

s a record of another anosmic rat which
ot die until 73 days after the start of
water. For the first 20 days it drank
ly more distilled water than heavy wa-
hen, over a long period of eratic behav-
finally drank heavy water in lethal
nts. Six rats showed the types of curves
n in Figs. 8B and C. They all finally
lethal amounts of heavy water. Of the
ts from which the olfactory bulbs were
ved after the differentiation had been
three failed to differentiate and died
13, 28, and 29 days; but three rats still
entiated. These last three anosmic rats
have still detected the presence of the
water in spite of changes in positions
bottles for fluids, use of entirely new
of bottles, and reoperations to make
in of removal of all olfactory tissue.
rats able to detect the difference in
ity between distilled water and heavy
? At room temperature heavy water is
more viscous than distilled water; at 5°
31% more viscous (2). Reactions to
ences in viscosity might be detected by
sensations (lingual nerves).
e differentiation experiment was con-
d on nine rats in a cold room (5°).
her experiment was conducted on six
t room temperature after sectioning of
ngual nerves and presumably eliminat-
ctile sensations.
ither experiment gave definite results.
sity could not play an important part
at 5° the rats failed to differentiate,
n part, also, to reduced aerosal in the
Thus, the ability of the three anosmic
o differentiate between heavy water
distilled water still remains unex-
ed.
cussion. Eratic variability of intake
similar conditions by rats puts heavy
in a category different from any other
ances that have been tested. Some rats
d to drink heavy water again after hav-
early shown a strong aversion for some
They did not, however, ingest lethal
nts, except in one instance.
ts avoid heavy water after a combina-
f two circumstances: (i) intake of high
nts of heavy water, high enough to
ce definite untoward symptoms after
24 hr on first exposure and (ii) exposure of the rat to enough heavy water aerosal to make an association between untoward symptoms and the smell of heavy water.

Thus, with the three exceptions, anosmic rats gave the same type of curves for heavy water that were obtained earlier (6) with the highly soluble and toxic chemicals sodium fluoroacetate (1080) and thallium sulfate. These two chemicals have no taste and no smell to either rats or human beings. This makes them such effective poisons. The rats drank them freely and were not able to distinguish them from distilled water. In the same way, anosmic rats drank heavy water freely and were unable to differentiate it from distilled water.

That 1080 and thallium sulfate have no taste to rats or man and probably to all other animals, was explained by the fact that animals have never been exposed to these chemicals in nature and so, in the process of evolution, have never built up an ability to protect themselves against them. Likewise, heavy water has never been present in nature except in the most minute amounts and it is only very slightly toxic anyway, so animals have never evolved an ability to taste it. That they are able to smell it is purely coincidental.

Summary. Restricted to heavy water (99.8%), rats drank it freely for the first day, then they drank progressively less and died within 14 days. When given a choice between distilled water and heavy water (99.8%) rats avoided heavy water, partly by virtue of some deleterious effects of heavy water and partly by virtue of a faint smell of heavy water with which the untoward effects could be associated. Rats did not taste heavy water. Whether a higher viscosity of heavy water plays any part could not be determined. Apparently the low intensity of any untoward effects of heavy water and the faintness of its smell could explain the erratic variability of the reactions of rats to heavy water that put it in a different category from other substances tested so far.

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nitate Incorporation in the Lungs of Dogs with Granulomatous Disease (39467)

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evious investigation of an animal model ffuse granulomatous lung disease has n that moderate hypoxia results in a iatic fall in oxygen utilization and a rise ctate production by the diseased lung). Newman and Naimark have found severe hypoxia reduces palmitate incor- tion by normal lung (5). Their study ipted us to test the effects of moderate xia upon this metabolic activity in the el of granulomatous disease.

thods. Pulmonary granulomatous dis- was produced by intravenous injection gs of 0.3 ml/kg complete Freund's ad- it (Difco Laboratories, Detroit, Mich.) successive days (1). The dogs were ed 3–4 weeks after injection when the se reaches its maximum extent. Eight al and eight diseased dogs were studied rding to an identical protocol. The dog thed the appropriate gas mixture (F_{IO_2} -0.25) until he attained a steady level ntilation. Five-tenths millicurie in 1.3 f crystalline [1-^{14}C]palmitic acid (New and Nuclear Corp., Boston, Mass.) then given intravenously. Arterial d samples were collected from the fem- artery at 20, 40, and 60 min for meas- ent of oxygen and carbon dioxide ten- and pH using an Instrumentation Labo- y blood gas analyzer. At 1 hr after nistration of labeled palmitate, the dog exsanguinated by transection of the ral arteries.

e lungs were promptly removed, ned, and immersed in 2:1 (v/v) chloro- -methanol at −20°, then homogenized, d, and filtered. The filtrate was washed 0.37% KCl (20 ml/100 ml filtrate), ifuged, and the supernatant was col- d. The washing was repeated twice with % KCl–chloroform–methanol solution volume ratio of 47:3:48, respectively. The washed lipid extract was dried and redissolved with petroleum ether to a volume of 500 ml. This comprised the stock solution from which all subsequent analyses were made. Four milliliters of the stock solution were dried under nitrogen, redissolved in chloroform, and put through 10-cm Kimax columns containing 2 g of silicic acid (325 mesh). Three subsequent washings of 10 ml of methanol eluted the phospholipids which were collected in preweighed vials, dried under nitrogen, and reweighed. Total lipid recovery from the columns, as determined by radioactivity recovered, was 80% for both the normal and diseased specimens.

Additional aliquots of the stock solution were similarly prepared for counting and fractionation. Counting was done with a Packard Tri-Carb liquid scintillation counter. Separation was done by thin-layer chromatography with silica gel H, using chloroform–methanol–water–glacial acetic acid, 80:25:4:1 (v/v) as the solvent. The plates were stained with iodine vapor and the spots were scraped separately for counting. In addition, the fatty acid composition of phosphatidyl choline was determined in four diseased and four control specimens by gas chromatography.

For autoradiographic studies two control and two diseased dogs were studied breathing room air according to the protocol previously described. One hour after infusion of 2 mCi/kg palmitic acid 9–10 ^{3}H (New England Nuclear Corp., Boston, Mass.) the lungs were removed and fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.6). Samples were postfixed in 1% osmium tetroxide, dehydrated in graded acetone solutions, and embedded in Araldite resin. One-micron-thick sections were dipped in Kodak Nuclear Track emulsion (NTB-2), air-dried, and incubated for 2, 4, and 6 weeks. The

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autoradiographs were developed in Kodak D-19 and examined by phase contrast and dark-field microscopy. For electron microscopic studies ultrathin sections were cut on a Sorval MT-2 microtome, stained with uranyl acetate and lead citrate, and examined in a Siemens Elmiskop 101.

Results. The data on lung weight and phospholipid content are summarized in Table I. On the average, there was a twofold increase in the lung weight to body weight ratio in the diseased dogs. This was attributable to the increase in weight of the diseased lung since body weight did not fall by more than 10% during the development of the pulmonary lesions. The larger standard deviations in the diseased group reflect the variability in extent of disease in different animals. Since phospholipid content increased linearly with increasing lung weight, as shown in Fig. 1, the phospholipid content per unit lung weight was, on the average, identical in both groups. The three diseased lungs which group with the normals in Fig. 1 showed features on gross examination characteristic of the granulomatous process. The lungs have a dark raspberry color and are filled with nodules which vary in size from 1 to 10 mm in diameter. Microscopically, the lesions are seen to be typical noncaseating granulomata (1). There are, in addition, increased numbers of free alveolar macrophages. Electron microscopy reveals an increased number of Type II pneumocytes lining all alveolar air spaces (Fig. 2). This proliferation of alveolar Type II cells is not localized to regions with granulomata, but is found as well in otherwise unaffected parts of the lung.

Autoradiographic studies reveal the developed silver grains clustered in the Type II alveolar epithelial cells in the diseased lung (Fig. 3). There is no concentration of silver grains over the granulomata or alveolar macrophages at 1 hr after infusion of labeled palmitate (Fig. 4). In the normal controls, the palmitate was similarly associated with the Type II alveolar pneumocyte and not with other normal cell types.

The uptake of labeled palmitate in total lung lipids was no different in diseased and normal groups, even during moderate hypoxia (Fig. 5). Nor was there any correlation between lipid counts per minute per lipid weight and P_{AO_2}, P_{aCO_2} or pH in either the normal or diseased groups.

There was no difference in the preferential incorporation of the label into the phosphatidyl choline fraction of phospholipids, being 57 ± 9% for diseased and 48 ± 13% for controls and no significant correlation between incorporation and P_{aO_2}, (Fig. 6). Accordingly, the data are summarized in Table II with reference only to the presence or absence of disease. The increase in phos-

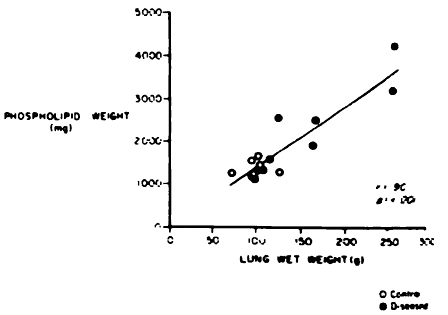

FIG. 1. Correlation of phospholipid content and lung wet weight in eight control (○) and eight diseased (●) dogs.

TABLE I. BODY, WET LUNG, AND PHOSPHOLIPID WEIGHTS (MEAN ± SD) IN EIGHT CONTROL AND EIGHT DISEASED DOGS.[a]

	Units	Controls	Diseased	*P*
Body weight	kg	12 ± 2	10 ± 2	NS
Lung wet weight	g	97 ± 17	162 ± 63	<.02
Phospholipid weight	g	1.3 ± 0.2	2.3 ± 1.0	<.02
Lung wet weight / Body weight	%	0.8 ± 0.1	1.7 ± 0.8	<.02
Phospholipid weight / Lung wet weight	%	1.4 ± 0.3	1.4 ± 0.3	NS

[a] The means are compared by the Student's *t* test of Fisher.

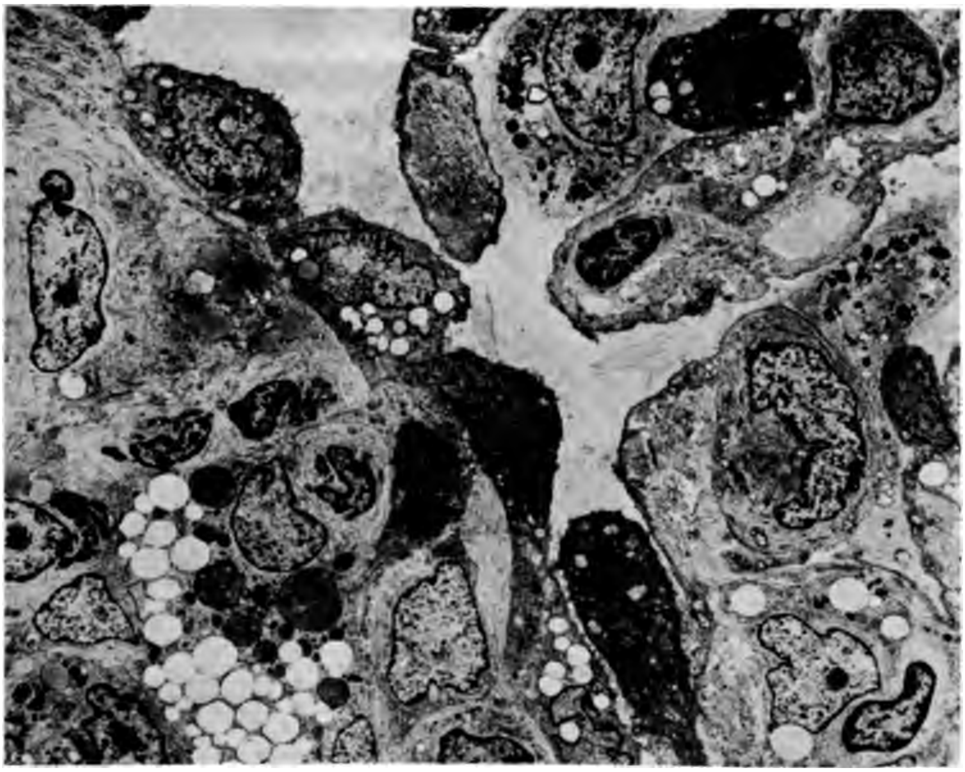

Fig. 2. This electron micrograph shows a portion of a granulomatous lesion encroaching on an air space. The lesion is made up of epithelioid cells containing fat, autophagosomes, and phagocytized material. The surface is lined by four adjacent Type II pneumocytes in different stages of development (×3000).

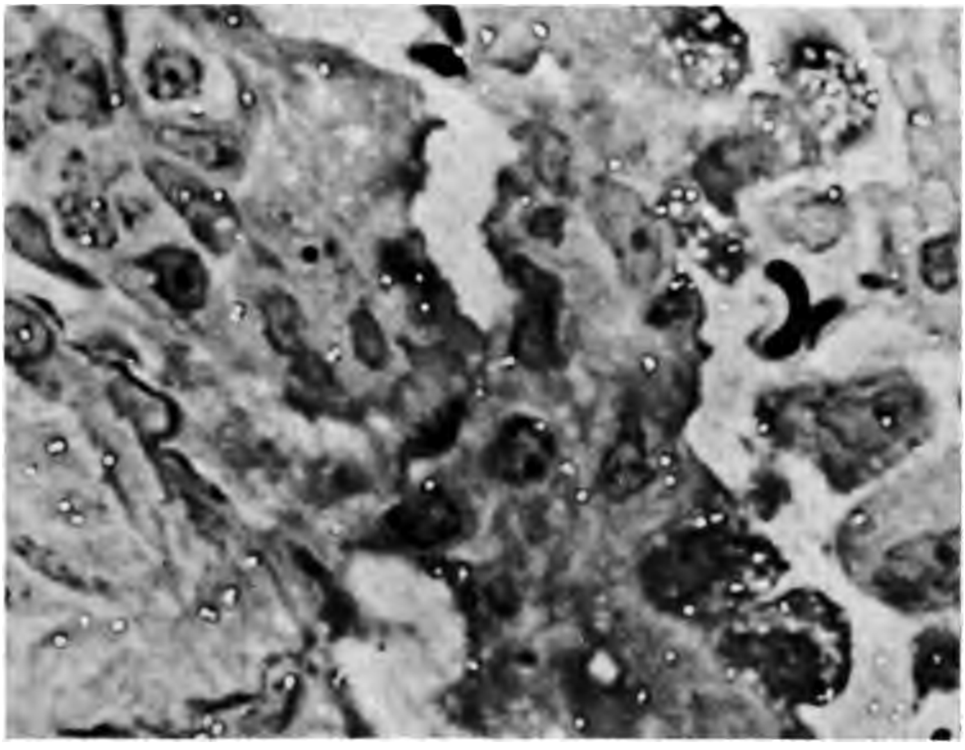

Fig. 3. In this autoradiograph examined in a phase contrast microscope, the developed silver grains are out of the plane of focus and are therefore seen as bright spots rather than dark grains. This micrograph shows a granulomatous lesion lined by Type II pneumocytes containing a concentration of silver grains. The dispersed grains in the rest of the field indicate the background level. (×750).

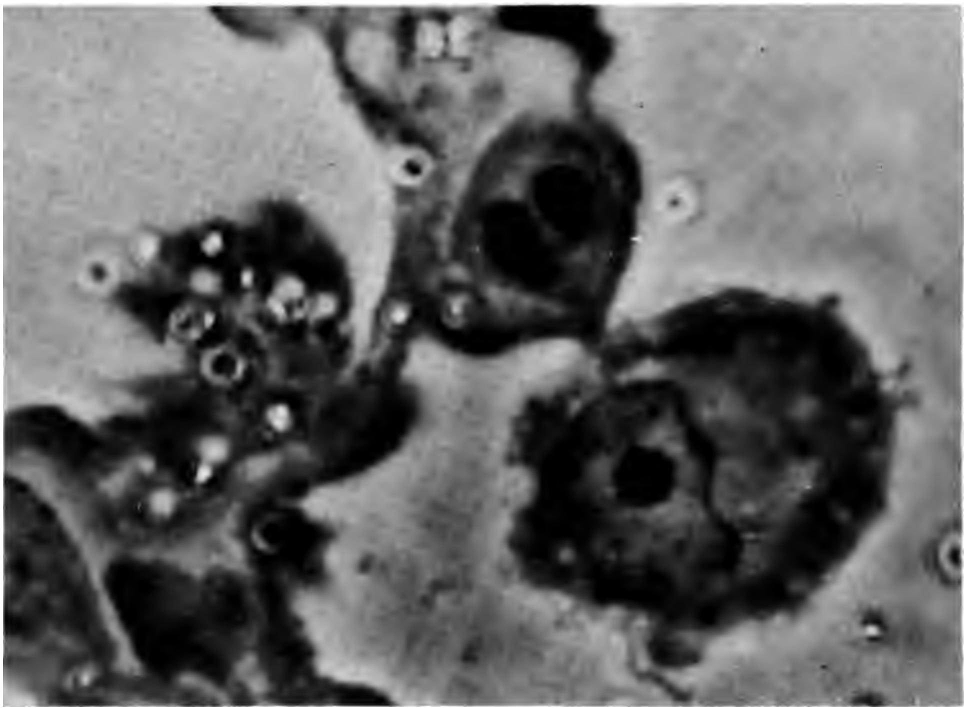

FIG. 4. In this autoradiograph examined by phase contrast at high magnification, the developed silver grains are seen as bright rings which are concentrated over a single Type II pneumocyte. An alveolar macrophage and an intracapillary polymorphonuclear leukocyte have no associated grains. (×2000).

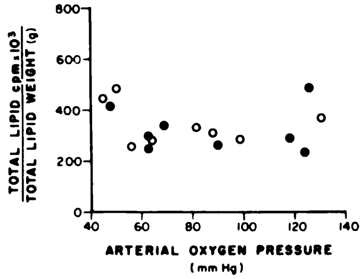

FIG. 5. Uptake of [1-^{14}C] palmitate in total lung lipids in eight control (○) and eight diseased (●) dogs.

pholipid counts in the diseased lung reflects the increase in lung weight; the specific activity, expressed as phospholipid counts per unit phospholipid weight, being no different in the groups.

Separation of the fatty acid constituents of the phosphatidyl choline fraction by gas chromatography in four control and four diseased dogs showed the C16 chain (palmitate) comprised, on the average, 59 and 57%, respectively, of the total fatty acids. Radioactivity recovered from the phosphatidyl choline fatty acids was almost exclusively confined to palmitate in both the normal (98%) and the diseased (94%) specimens.

The total neutral lipid count per gram

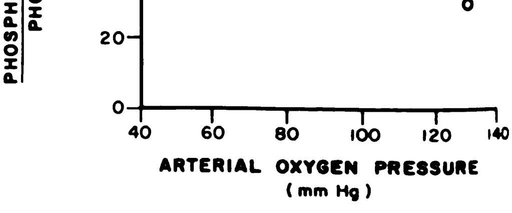

FIG. 6. Incorporation of [1-^{14}C] palmitate into phosphatidyl choline fraction of lung phospholipids in eight control (○) and eight diseased (●) dogs.

E II. Incorporation of [1-^{14}C]Palmitate into Lung Phospholipids of Eight Control and Eight Diseased Dogs (Mean ± SD).[a]

	Units	Controls	Diseased	P
jectate Dose	cpm × 10^6	43 ± 14	38 ± 20	NS
ospholipid Counts	cpm × 10^3	488 ± 213	794 ± 238	<.02
$\frac{\text{lipid counts}}{\text{lipid weight}}$	$\frac{\text{cpm} \times 10^3}{\text{g}}$	369 ± 135	380 ± 166	NS
$\frac{\text{tidyl choline counts}}{\text{pholipid counts}}$	%	48 ± 14	55 ± 9	NS

means are compared by the Student's *t* test of Fisher.

l lipid weight was 127 ± 58 in normal nd 103 ± 29 in the diseased lungs. Of tal neutral lipid counts per minute, akdown as a percent found in choles- fatty acids, triglycerides, and choles- sters was 14, 9, 54, and 14, respec- for normals and 18, 3, 59, and 9 for eased. The differences in breakdown n normal and diseased were not sta- ly significant.

ussion. The present study confirms observations that palmitate is incor- d preferentially into the phosphatidyl fraction of the phospholipids of the Our results in normal lungs demon- hat the incorporation of the fatty acid in the Type II alveolar pneumocyte, been shown previously by one of us H.) as well as by others (6, 7). Sur- ly, the presence of granulomatous does not interfere with the incorpo- of palmitate. The increase in lung in this disease results from an in- in cell mass; there are no differences atios of protein weight: wet weight or eight:dry weight between diseased nd normal lungs (4).

e there is a proliferation of epithelioid ononuclear inflammatory cells in the omas which are not known to incor- palmitate one might expect there be a reduction in the specific activity led palmitate (counts per minute per f phospholipid) in the diseased lung. , there is an increased uptake of pal- in proportion to the increase in of the lung in the diseased group. ssibility that this behavior of the dis- lung might be due to a reduction in culating levels of palmitate was considered, but measurement revealed no differences in plasma concentrations of palmitate or albumin between the diseased and control dogs. Since we have identified an increase in numbers of Type II alveolar pneumocytes in the diseased lungs, and found by autoradiographic study that labeled palmitate is preferentially incorporated into these cells, we conclude that the enhanced incorporation of fatty acid in the diseased lung is the result of metabolic activity of the Type II alveolar cells. The autoradiographic studies identify primarily palmitate incorporation into phospholipids rather than neutral lipids, since acetone used in the preparation of these tissues may have extracted the latter. Elsbach (8) and Mason and co-workers (9) have shown that the alveolar macrophage incorporates palmitate into phospholipids and these cells are increased in adjuvant induced pulmonary disease. Yet, Young and Tierney (10) and Thomas and Rhoades (11) have reported that it requires more than 6-hr after intravenous infusion of radioactive palmitate before the peak of specific activity is found on the surface of the alveoli in normal lungs. In addition, the report of Askin and Kuhn (7) confirms that in normal lungs the label first appears in the Type II alveolar cells. Hence, it seems unlikely that the label would be found in the free alveolar cells in the present study where the lungs were harvested 1 hr after palmitate infusion. This time was chosen because other studies have shown that the maximal uptake of palmitate in normal lung occurs within this period of time (4, 5, 11).

Whereas Newman and Naimark (5) demonstrated an effect of severe hypoxia on palmitate incorporation by normal lung in the rat at an arterial tension of 29 mm Hg, the present study failed to demonstrate such an effect over a range of 40–140 mm Hg in the dog. We believe this difference reflects the level of hypoxia studied. While fatty acid incorporation appears to be relatively unaffected by moderate hypoxia, dramatic changes in pulmonary oxygen utilization and lactate production have been previously demonstrated during hypoxia in this disease model (1–4).

Summary. Moderate hypoxia did not influence the pulmonary incorporation of an intravenous dose of [1-^{14}C]palmitate either in dogs with experimentally produced granulomatous disease or in normal controls. The lung weight in the diseased animals, was, on the average, double that of the controls. There was a proportionate increase in uptake of the radioactive label at 1 hr after infusion in the diseased lungs, hence the specific activity of labeled palmitate (counts per minute per gram of phospholipid) was no different in the two groups. Moreover, half the radioactivity of the phospholipids was recovered in palmitate separated from the phosphatidyl choline fraction in both diseased and normal lungs. Anatomic studies demonstrated increased numbers of Type II pneumocytes lining all alveolar air spaces in the diseased lung. Autoradiographic studies indicated the presence of labeled palmitate in the Type II cells, but not in the inflammatory cells of the granulomata. We conclude that the increased palmitate uptake in this disease in accounted for by the metabolic activity of the Type II pneumocytes.

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Corticotropin Releasing Factor (CRF) Activity in Rat Plasma[1] (39468)

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e have been several reports of corti- n releasing activity in extrahypothal- ssues and fluids (1-6). Indirect evi- f extrahypothalamic CRF was pro- n cross-circulation experiments con- in rats by Brodish and Long (2). indirect evidence of extrahypothal- tivity was also established by Egdahl) found that dogs with pituitary is- ontinued to secrete ACTH despite l of the entire nervous system.

:t evidence for extrahypothalamic ' CRF was first shown by Lyman- and Brodish (7) who found that pe- l plasma taken from stressed, hy- ectomized rats with median emi- esions had greater CRF activity than from similar nonstressed animals. In periments CRF activity of the donor ma was assayed in median eminence- l animals which had been shown to l to median eminence extract but not ral cortical extract or to ether stress. ssue CRF and hypothalamic CRF milar in that both stimulated corti- retion in assay rats. However, tissue d hypothalamic CRF differed in the urses of effect in the assay rats. Hy- mic CRF activity provoked a tran- rly effect whereas the effect of tissue as found to be prolonged.

a has also been reported to stimu- TH release from rat pituitary cells ned in monolayer culture (6). As 0.1% v/v plasma in the assay system tes ACTH release.

g the course of studies of hypotha- RF activity we observed a similar f peripheral plasma on incubated, d pituitary cells prepared by the of Swallow and Sayers (8). The present report describes efforts to further characterize plasma CRF activity.

Preparation of isolated pituitary cells. Four to six male Sprague-Dawley rats, weighing 200-300 g were used in each experiment. After decapitation the anterior pituitaries were removed, rinsed in normal saline, blotted, quartered, and placed in a 50-ml Erlenmeyer flask containing 20 ml of Krebs-Ringer bicarbonate buffer with 0.2% glucose (KRBG) and 0.5% trypsin (Worthington Biochemical Corp., Freehold, N.J.) (8). The tissue pieces were agitated at 37° in a 95% O_2:5% CO_2 atmosphere. After 20 min the supernatant containing dispersed cells was transferred to an iced flask, fresh KRBG with trypsin was added to the remaining fragments and the above process was repeated. After three washes, the combined supernatants were centrifuged at 100*g* for 30 min at 4°. The pellet containing the cells was resuspended in KRBG containing 1% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) and 30 mg of lima bean trypsin inhibitor (Worthington Biochemical Corporation, Freehold, N.J.), yielding a final concentration of 0.5 pituitary equivalents per milliliter. Cell suspension aliquots of 0.5 ml containing approximately 10^5 cells were preincubated in 10-ml Teflon beakers for 10 min at 37° until the test substances were added.

Preparation and additions of test substances. The following test substances were added: lyophilized rat hypothalamic extract (RHE) (NIAMD-rat HE-RP-1), rat plasma or serum, heparin, and rat plasma fractionated as described below. All test substances were adjusted to 500-μl volumes with KRBG and added to the cell suspension, yielding a final incubation volume of 1 ml. RHE was dissolved in 2 ml of 0.2 *N* acetic acid, aliquoted into 100-μl vials, and frozen until use. The aliquots were thawed and diluted with buffer before addition to the test system. Blood was collected from rats

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by rapid decapitation (unstressed) or by exsanguination after 5 min exposure to ether (stressed). Samples were collected on ice, centrifuged at 100*g*, and frozen until use. The possible CRF activity of heparin (sodium heparin, Invernex, 1000 USP units per milliliter) was tested by introduction into the cell incubation at the same concentration as that used to heparinize blood (10 units/ml).

Fractionation of plasma. Two methods were employed (i) 2 ml of plasma was introduced onto a 1.5 × 30-cm G-75 Sephadex column and eluted with an 0.05 *M* phosphate buffer (pH 7.4) at 25°. (ii) 10 ml of plasma separated by vacuum ultrafiltration at 50 Torr at 4° using a membrane with an average molecular weight retention of 15,000. Both the retained fraction (retenate) and the ultrafiltrate were collected and stored at 4° or until assayed. In both experiments a portion of the raw plasma was assayed to determine recovery.

Assay of ACTH. ACTH released into the incubation medium was determined by radioimmunoassay as previously described (9). The antibody employed is highly specific for α_h-ACTH and α^{11-24} ACTH; reacting with both on an equimolar basis. It fails to cross-react significantly with the following polypeptides: α-MSH, β_p-MSH, β_h-MSH, $\alpha_p{}^{17-39}$ACTH, $\alpha_h{}^{17-39}$ACTH, and $\alpha_p{}^{25-39}$ ACTH and $\alpha_h{}^{25-39}$ ACTH or α^{1-16NH_2} ACTH. The sensitivity limit of the assay is 30 pg/ml. In the present studies the measured quantities of ACTH were in the nanogram per milliliter range.

Results. Dose-response relationships in the isolated pituitary cell system were established using the standard NIAMDD rat hypothalamic extract as a stimulus. The results of this experiment are shown in Fig. 1. The lower asymptote of the dose-response curve was used to estimate the sensitivity of the assay system, which was about 0.01 mg RHE. Maximal stimulation was achieved by 1.0 mg RHE which caused release of 10 ng ACTH/10^5 cells during the 30 min incubation period. The ACTH-releasing activity of 0.1 mg RHE was chosen as a standard for the studies.

The time course of ACTH release in response to 0.1 mg RHE is depicted in Fig. 2. Approximately one-half of the total net ACTH release occurred within 5 min after addition of the RHE as previously observed by Takebe *et al.* using the monolayer culture technique (6). After 30 min, 85% of the total net ACTH release had occurred and this duration of incubation was routinely employed. It was determined that a 5-min period of stabilization of the system before the addition of the test substance (preincubation period) was technically convenient.

The stimulatory effects of graded volumes of rat plasma and serum are shown in Table I. Both serum and plasma produced dose-related ACTH release. Although rat plasma had approximately twice the ACTH releasing potency of serum at the highest tested dose the differences were not statistically significant. Since the plasma was prepared with heparin and the serum was not, the possible ACTH-releasing effect of heparin was assessed by testing heparin alone and by heparinizing serum. In two experiments

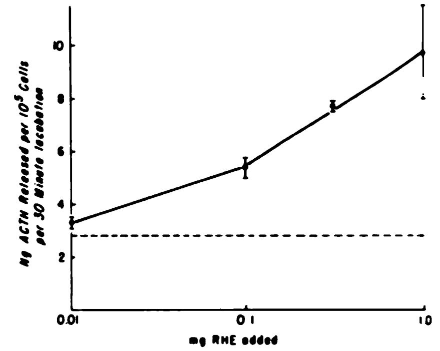

FIG. 1. Dose-response effect of rat hypothalamic extract (RHE) on ACTH release from dispersed pituitary cells.

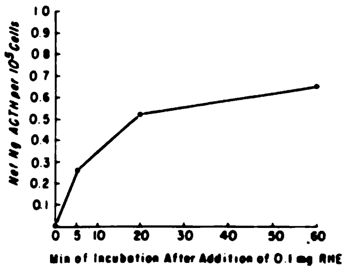

FIG. 2. Time-course of rat hypothalamic extract (RHE)-induced ACTH release by dispersed pituitary cells.

LE I. Effect of Rat Plasma and Serum on in Vitro Pituitary ACTH Release.

t substance	Amount added (μl)	Number of replications	Net ACTH release[a] (ng/10^6 cells/30 min)
	100	8	4.7 ± 1.4
	10	6	1.1 ± 0.6
	1	4	0.5 ± 0.4
	100	3	1.9 ± 1.8
	10	2	1.5, 0
	1	2	0, 0
(unstressed)	100	4	5.3 ± 2.3
(stressed)	100	4	4.7 ± 1.7

:an ± SEM.

:in alone had no ACTH-releasing ac- (data not shown).

four experiments the effects of plasma ned from stressed and unstressed rats compared. As indicated in Table I produced ACTH release, but the dif- ce between the two was not significant. e ACTH-releasing activity of plasma urther characterized by ultrafiltration gel separation. After ultrafiltration us- membrane with an average molecular it retention of 15,000, 93% of the H releasing activity was found in the tate as shown in Table II. Plasma was separated by using G-75 Sephadex and :ollected fractions tested for ACTH- sing activity (Fig. 3). The fractions with H-releasing activity were not retarded e Sephadex. The findings of these stud- ggest that the CRF activity of plasma is iated with a molecular weight greater 15,000.

cussion. These studies confirm the nce of an ACTH-releasing substance plasma and suggest it is, or is associ- with, a large molecule. Both stressed nstressed rat plasma and serum caused H release from dispersed rat pituitary supporting the observation of Takebe (6) who used a pituitary monolayer cell re system. The present studies extend observations by showing with two dif- t separation methods, that the sub- e in plasma behaves like a molecule of lecular weight of greater than 15,000. e CRF activity of rat plasma (and se- is like hypothalamic CRF in that the pituitary response is dose-related. However, it is apparently not identical to hypothalamic CRF because the dose-response curves of the two materials were found to be different in Takebe's studies (6) and because the CRF activity in plasma is not altered by factors ordinarily expected to affect pituitary-adrenal function, such as stress. Finally, it can be calculated that the plasma compartment contains many more times the CRF activity than that of the hypothalamus. In the present studies as little as 100 μl of rat plasma had approximately the same ACTH releasing activity as the equivalent extract of 0.5 rat hypothalamus. The above data would suggest a nonhypothalamic source of plasma CRF.

It is also possible that plasma CRF is a purely artifactual phenomenon. Both the dispersed pituitary cell technique of Sayers and the monolayer culture technique employ cells that are exposed directly to the stimulatory agent. Under these circumstances the cells do not have the protection normally afforded by the selective barrier of

TABLE II. The Effect of Rat Plasma Fractionated by Ultrafiltration on *in Vitro* Pituitary ACTH Release.

Fraction	Volume added (μl)	Net ACTH released[a] (ng/10^6 cells/30 min)	
		Experiment 1	Experiment 2
Dialysate	100	1.1	1.2
	10	0	1.0
	1	0	0
Retentate	100	13.2	9.7
	10	5.7	3.2
	1	1.0	1.2

[a] Results of two experiments.

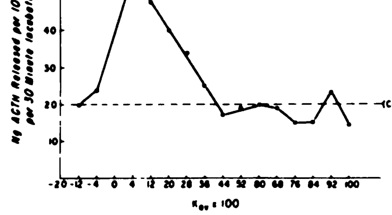

Fig. 3. Effect of G-75 Sephadex-fractionated rat plasma on dispersed pituitary cells.

the capillary wall and associated structures between plasma and the secretory cell and the plasma. Thus, plasma may exert a nonphysiological effect directly on the cells. This explanation would account for the apparent discrepancy between the relative potencies of hypothalamic CRF and plasma CRF. Furthermore, the explanation might account for the apparently previously inexplicable phenomenon of Egdahl (3) in which dogs with isolated pituitaries following complete brain removal were found to elaborate massive amounts of ACTH. It is possible that the brainectomized dog has increased pituitary capillary permeability and plasma factors not ordinarily reaching the pituitary cells, such as plasma, may come into direct contact with cells and cause ACTH release. The biochemical nature of plasma CRF and its physiological and/or pathophysiological role awaits further study.

Summary. The ACTH-releasing activity of hypothalamic extract and rat plasma was examined with the dispersed rat pituitary cell technique of Swallow and Sayers (8). Although both plasma and serum caused ACTH release which was dose-related, stress did not enhance the ACTH releasing activity. Furthermore, separation studies of plasma using ultrafiltration and gel separation suggest that the CRF activity in plasma is associated with molecules of a molecular weight greater than 15,000.

The hypothalamic extract used in this study was kindly provided by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD) Rat Pituitary Hormone Distribution Program.

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calorie	cal	millimeter	mm
centimeter	cm	milliosmole	mOsm
counts per minute	cpm	minute	min
cubic centimeter	cm^3	molal (concentration)	*m*
Curie	Ci	molar (concentration)	*M*
degree Celsius (Centigrade)	-°	mole	spell out
degree Fahrenheit	°F	molecular weight	mol wt
diameter	dia	nanogram	ng
gram	g	nanometer	nm
hour	hr	normal (concentration)	*N*
inch	in.	osmole	Osm
inside diameter	i.d.	ounce	oz
intramuscular	im	outside diameter	o.d.
intraperitoneal	ip	parts per million	ppm
intravenous	iv	percent	%
kilocalorie	kcal	picogram	pg
kilogram	kg	revolutions per minute	rpm
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